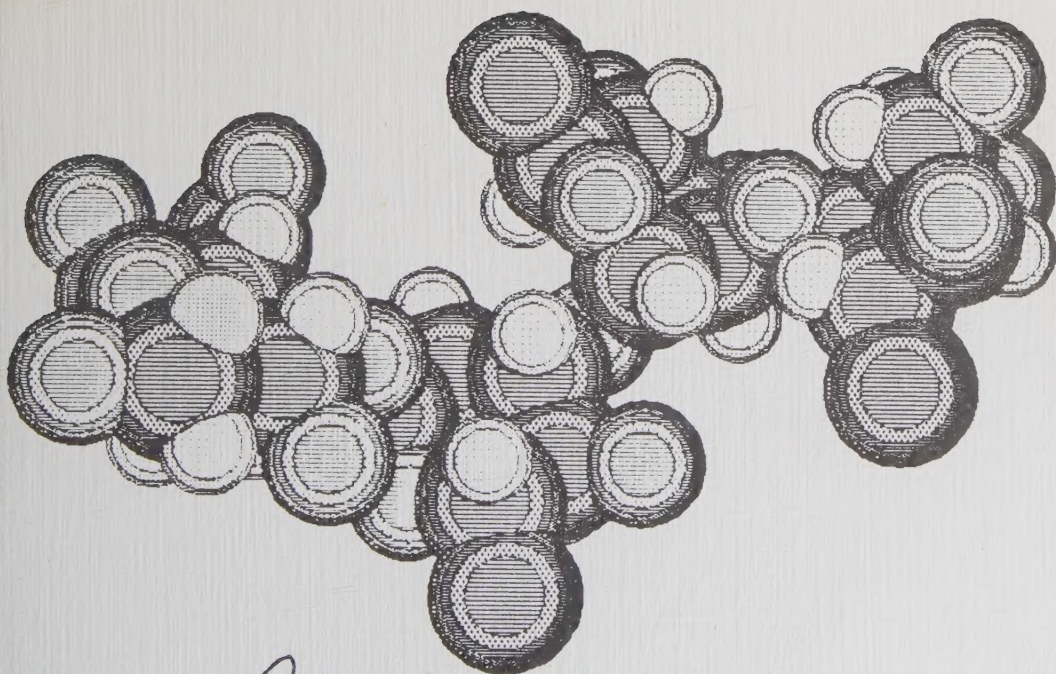


TRIFLUOROACETOLYSIS

Specific techniques for the isolation of biologically
active oligosaccharides from glycoconjugates

ALF GUNNARSSON



Diss. Runcel.

1984



TRIFLUOROACETOLYSIS

Specific techniques for the isolation of biologically
active oligosaccharides from glycoconjugates

av

ALF GUNNARSSON

Civ ing, Ög

Akademisk avhandling som för avläggandet av teknologie
doktorsexamen vid Tekniska fakulteten vid Lunds
Universitet kommer att offentligen försvaras i Sal B,
Kemicentrum, Lund, fredagen den 1 juni 1984 kl 10.00

TYP AV DOKUMENT ☒ Doktorsavhandling ☐ Examensarbete ☐ Kompendium	☐ Ansökan ☐ Reserapport ☐ Delrapport ☐ Slutrapport	☐ Tidskriftsartikel ☐ Konferenssuppsats ☐	DOKUMENTBETECKNING/CODEN LUTKDH/(TKOK-1014/1-120/(1984)
AVDELNING/INSTITUTION Avd. för kolhydratkemi, Kemicentrum, Lunds Universitet, Box 740, 220 07 Lund			
FÖRFATTARE Gunnarsson, Alf			
DOKUMENTTITEL OCH UNDERTITEL TRIFLUOROACETOLYSIS: Specific techniques for the isolation of biologically active oligosaccharides from glycoconjugates.			
SAMMANFATTNING The aim of this work was to extend the role of trifluoroacetolysis for isolation and characterization of biologically active carbohydrates from glycoconjugates. Hexuronic acids are degraded by trifluoroacetolysis if they contain a free carboxylic acid group and if they are incapable of forming 6,3-lactones. These features were used in development of a specific elimination procedure of reducing 4-O-substituted hexose residues and in isolation of oligosaccharides from the carbohydrate - protein linkage region in chondroitin 4-sulphate. Glycosphingolipid models were used to investigate the trifluoroacetolysis conditions required for isolation of the oligosaccharide chains. Oligosaccharides could be isolated quantitatively from glycosphingolipids containing an unsaturated sphingosine. Structural studies were carried out on oligosaccharides released from glucoamylase G1 and glycosphingolipids from sheep erythrocyte membranes. The released oligosaccharides from glycosphingolipids from sheep erythrocyte membranes were tested as inhibitors of the haemagglutination of sheep erythrocytes by <u>Staphylococcus saprophyticus</u>.			
NYCKELORD Trifluoroacetolysis, biologically active carbohydrates, glycoconjugates, isolation, structure.			
DOKUMENTTITEL OCH UNDERTITEL - SVENSK ÖVERSÄTTNING AV UTLÄNDSK ORIGINALTITEL TRIFLUOROACETOLYS: Specifika tekniker för isolering av biologiskt aktiva oligosackarider från glykokonjugater.			
TILLÄMPNINGSOMRÅDE Isolering och karakterisering av biologiskt aktiva kolhydrater från glykokonjugater. De isolerade oligosackariderna kan utnyttjas i konformationsstudier och som modell substanser i biologiska system för att öka förståelsen för glykokonjugaters biologiska roll.			
NYCKELORD Trifluoroacetolys, biologiskt aktiva kolhydrater, glykokonjugater, isolering, struktur			
UTGIVNINGSDATUM år 1984 mån juni	ANTAL SID (inkl bilagor)	SPRÅK ☐ svenska ☒ engelska ☐ annat	
ÖVRIGA BIBLIOGRAFISKA UPPGIFTER			ISSN
			ISBN
			PRIS
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TRIFLUOROACETOLYSIS

Specific techniques for the isolation of biologically
active oligosaccharides from glycoconjugates

by

ALF GUNNARSSON



LUND 1984

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LIST OF PAPERS

This thesis is based on the following papers referred to by Roman numerals in the text.

- I. The reactivity of uronic and aldonic acid derivatives towards trifluoroacetolysis. A new method for specific elimination of reducing 4-O-substituted hexose residues.
A. Gunnarsson and S. Svensson
Carbohydr. Res., in press.
- II. Degradation of chondroitin 4-sulphate by trifluoroacetolysis. Isolation of oligosaccharides from the carbohydrate - protein linkage region.
A. Gunnarsson, S. Svensson and L. Rodén
Carbohydr. Res., in press.
- III. Specific cleavage of the glycosidic bond between the carbohydrate and ceramide portions in glycosphingolipids using trifluoroacetolysis.
A. Gunnarsson, J. Lundsten and S. Svensson
Acta Chem. Scand., in press.
- IV. Structural studies on the O-glycosidically linked carbohydrate chains of glucoamylase G1 from Aspergillus niger.
A. Gunnarsson, B. Svensson, B. Nilsson and S. Svensson
Eur. J. Biochem., submitted.
- V. Oligosaccharide structures mediating agglutination of sheep erythrocytes by Staphylococcus saprophyticus.
A. Gunnarsson, P.-A. Mårdh, A. Lundblad and S. Svensson
Infect. Immun., submitted.

Footnote

All sugars, when not indicated, are assumed to have D-configuration and to be in pyranose form.

1. INTRODUCTION

Carbohydrates, together with proteins and lipids, are Nature's most abundant organic compounds. The monosaccharide glucose, and its polymers starch and glycogen play a central role in cellular metabolism, especially in the storage and utilisation of energy. Other polysaccharides, of which cellulose is an example, have a more structurally orientated function, being major cell wall components in plants and microorganisms. In the animal kingdom the cell membranes are lipid bilayers with a great variety of carbohydrate structures embedded in them in the form of glycoproteins and glycosphingolipids (Fig. 1). Such hybrids of carbohydrates and proteins or lipids are known as glycoconjugates.

PLASMA MEMBRANE

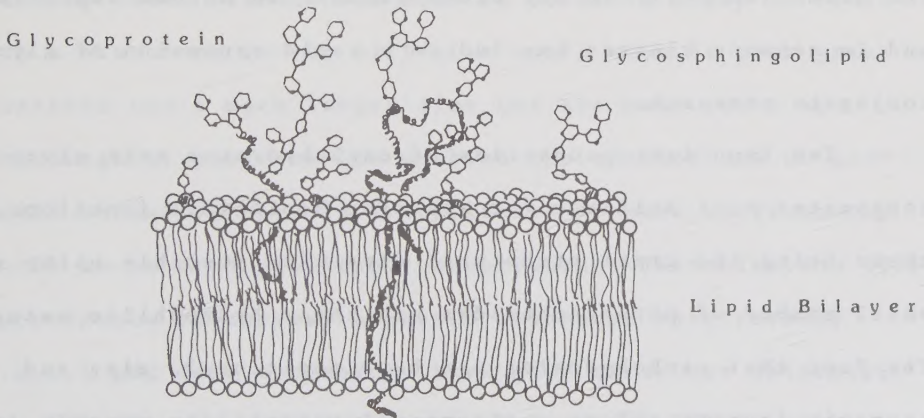


Fig. 1

Schematic representation of glycoconjugates in a cell membrane illustrating that the complex oligosaccharide structures appear to extend from the cell surface into the extracellular space.



Most proteins have a carbohydrate moiety which can vary from about 1% to over 85% of the molecule. The carbohydrate chains of glycoconjugates are polyglycosides and in glycoproteins they are attached glycosidically to amino-, thiol- or hydroxyl-groups in the polypeptide chains (1,2). Proteoglycans are a specialised class of glycoproteins containing acidic, frequently sulphated sugars. The oligosaccharide chains are either O- or N-glycosidically linked to the protein (3). In glycosphingolipids the carbohydrate part is O-glycosidically linked to a hydrophobic moiety (ceramide) comprising a long-chain base and a fatty acid residue (4,5). Glycosphingolipids with as many as 59 sugar units have recently been reported (6).

Over the past fifteen years the biological role of glycoproteins and glycosphingolipids has increasingly been recognized (1,2,7-9). The importance of these glycoconjugates in such different locations as, for example, the surface of the mammalian cell, in the blood plasma, in mucous secretions and in nervous tissues has led to a rapid expansion of glycoconjugate research.

Two important properties of carbohydrates make glycoconjugates most suitable for specific biological functions, these being the great structural diversity possible using a small number of monosaccharides and their hydrophilic nature. The fact that carbohydrates can have positional, ring and anomeric isomers allows a structural versatility greatly in excess of that possible with peptides. For example, a trisaccharide consisting of three different hexopyranosides can form over 1000 isomers whereas a tripeptide can form only 6

isomers (10). The hydrophilicity of carbohydrates, as constituents of glycoconjugates in cell membranes, will orientate them away from the hydrophobic membrane lipid bilayer (Fig. 1) and ensure that the carbohydrate parts are the most distal molecular structures encountered by any reagent approaching a membrane surface. These properties make the glycoconjugate carbohydrates well suited to provide a variety of different recognition signals. The biological function of glycoconjugates has been demonstrated in many reviews (1,2,7-9,11-16). Glycoconjugates function as cell surface antigens, as receptor sites for viruses, bacteria, bacterial toxins, enzymes, hormones and proteins, and in cell growth regulation and differentiation.

In order to understand the biological function of the glycoconjugates there is a need for methods to isolate and characterize glycoconjugate carbohydrates. For this, chemical, physical and enzymatic methods are available (17-19). Refining these methods has made it possible today to elucidate a structure in the milligram scale when it took grams of substance and a much longer time not too many years ago. Technological progress has enabled the application of gas-liquid chromatography (g.l.c.) linked to mass spectrometry (m.s.) to be used profitably in structure determination (20). Improvements in nuclear magnetic resonance spectroscopy (n.m.r.) and data technology have made structure determination (21,22) and conformational studies (23,24) of carbohydrates more accessible.

Trifluoroacetolysis is a chemical method used to isolate and characterize oligosaccharide chains in glycoproteins

(25-28) and glycosphingolipids (29,30). By varying the trifluoroacetolysis conditions specific oligosaccharides can be isolated from the glycoconjugates.

This thesis extends the role of the trifluoroacetolysis procedure in isolation and characterization of glycoconjugate carbohydrates. Special interest has been focussed on the reactivity of acidic carbohydrates towards trifluoroacetolysis. Furthermore, reaction conditions for the cleavage of the carbohydrate-ceramide bond have been investigated and used in structural studies.

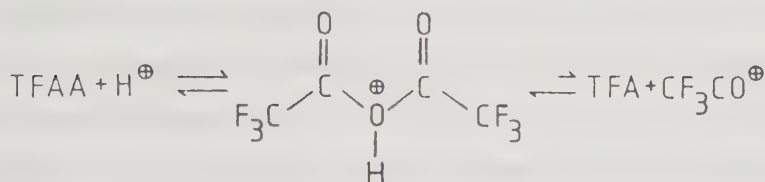
2. TRIFLUOROACETOLYSIS

2.1 Introduction

Trifluoroacetolysis is carried out in mixtures of trifluoroacetic acid (TFA) and trifluoroacetic anhydride (TFAA) in various proportions and at different temperatures. The stability of glycosidic bonds under trifluoroacetolysis is due to the strong electron withdrawing effect exerted by the O-trifluoroacetyl groups introduced into the sugar moieties (31-36). The trifluoroacetolysis mixture is capable of converting 2-acetamido-2-deoxy sugars into 2-deoxy-2-trifluoroacetamido sugars by transamidation (37).

The trifluoroacetolysis conditions used are all capable of transamidation and the ratio TFA/TFAA is varied from 1:1 to 1:100 at 100° for 48 h. The formation of what is pro-

bably the reactive species in trifluoroacetolysis, the trifluoroacetylum ion, can take place as depicted in Scheme 1.



Scheme 1

Numerous model compounds have been subjected to trifluoroacetolysis to investigate their stability. Most reducing sugars (31) and glycosides (32) that have been tested are stable as their pertrifluoroacetylated derivatives. Investigations on the stability of partially methylated methyl glycosides towards trifluoroacetolysis showed that 2-O-, 4-O- and 6-O-trifluoroacetyl groups are most effective in preventing solvolysis of the glycosides (33-36). Complete degradation of reducing 2-acetamido-2-deoxy sugars at 1:1 TFA/TFAA enabled development of a method for elimination of such residues at the reducing end of oligosaccharides (31). The aglycone structure has little effect on the stability of the glycosidic bond to trifluoroacetolysis (36).

2.2 Isolation of the oligosaccharide chains of glycoconjugates

A prerequisite for the understanding of the role of

carbohydrates in the biological function of glycoconjugates is a knowledge of their structure. A number of methods are available for the isolation of carbohydrate chains in glycoproteins, including hydrazinolysis (38) and alkaline borohydride treatment (39) for N-glycosidically-linked carbohydrate chains, and mild alkaline treatment for carbohydrate chains O-glycosidically linked to serine or threonine (40). Oligosaccharide chains in glycosphingolipids can be isolated by oxidative cleavage of the double bond in the acetylated sphingosine (long-chain base) moiety followed by alkaline treatment (41).

Trifluoroacetolysis is a powerful additional method for isolation of oligosaccharide chains in glycoconjugates and has the advantage that the same procedure can be used for the simultaneous isolation of the great majority of the carbohydrates found in glycoconjugates. N-Glycosidically-linked oligosaccharides in glycoproteins are cleaved off by transamidation with concomitant degradation of the protein part (25,27) and O-glycosidically-linked carbohydrates are released by acid catalyzed elimination from serine and threonine residues (28). Isolation of carbohydrate chains from glycosphingolipids by trifluoroacetolysis is an equally facile process, but requires an unsaturated sphingosine base (30).

2.3 Trifluoroacetolysis - mass spectrometry

N-Trifluoroacetyl derivatives have a number of features which make them suitable for g.l.c.-m.s. analysis as per-

methyiated derivatives. Trifluoroacetolysis will effect transamidation of the N-acetyl groups in oligosaccharides, a process which increases their volatility. Since the limiting factor in g.l.c. analysis is often a compound's volatility, trifluoroacetolysis enables the analysis of larger hexos-amine-containing oligosaccharides by g.l.c.-m.s. as their permethylated derivatives.

Up to now six hexose units (including N-trifluoroacetyl hexosyl residues) can be analysed by g.l.c.-m.s. The restriction is the upper temperature limit for the g.l.c. columns. In the future, the expected improvements in column materials, should allow analysis of mixtures containing larger oligosaccharides by g.l.c.-m.s.

The characteristic fragmentation in m.s. of oligosaccharides containing 3-O- or 4-O-substituted 2-deoxy-2-trifluoroacetamido sugars make sequence determination easier and more reliable (30,42). The fragmentation in m.s. of methyl glycosides of N-trifluoroacetyl derivatives has been investigated in detail (43) and the results simplify structural analysis of N-acetyl-containing oligosaccharides released from glycoproteins and glycosphingolipids by trifluoroacetolysis.

These developments will improve the techniques available for the characterization of complex oligosaccharides in glycoconjugates and hopefully lead to a better understanding of their biological function.

3. CHARACTERIZATION OF BIOLOGICALLY ACTIVE CARBOHYDRATES BY TRIFLUOROACETOLYSIS

3.1 Trifluoroacetolysis of acidic carbohydrates

3.1.1 General

A wide variety of naturally occurring compounds contain acidic carbohydrates. Sialic acid (NeuNAc) derivatives are present as terminal sugar residues in glycoproteins and gangliosides. Uronic acid residues are constituents of polysaccharides in proteoglycans and bacterial cell walls.

Sugar residues containing acidic functions express chemical reactivities different from the corresponding neutral derivatives. For example, the glycosidic linkage of NeuNAc is more susceptible to acid hydrolysis than the glycosidic linkages of neutral hexoses, and thus asialo glycoconjugates can be prepared easily by mild acid hydrolysis (44). Methyl glycopyranosiduronic acids are hydrolysed at a slightly lower rate than the corresponding methyl glycopyranosides (45) and this lower rate of hydrolysis of hexuronic acids has been used in the isolation of acidic oligosaccharides by partial acid hydrolysis of bacterial cell wall polysaccharides (46).

In many polysaccharides the uronic acid is 4-O-substituted. This feature can be used specifically to eliminate 4-O-substituents of uronic acids in proton poor medium (β -elimination) (47). Determination of the attachment sites of hexuronic acids by a modified alkaline degradation has

also been reported (48,49).

Naturally occurring carbohydrates containing aldonic acids are as yet unknown. However they have synthetic applications, for example in synthesis of ascorbic acid (vitamin C) and furthermore exist as degradation products in strong basic treatment of reducing sugars (50).

In proteoglycans a number of polysaccharide chains are covalently linked to a protein core. Even though the proteoglycans are a specialised class of glycoproteins, there are considerable differences in the structure of the polysaccharide chains. The polysaccharides of proteoglycans consist of a repeating disaccharide unit containing one hexosamine. Uronic acids and sulphate ester groups are very common in proteoglycans with two exceptions, keratan sulphate and hyaluronic acid, respectively (3).

Xylose, O-glycosidically linked to serine, is specific for connective tissue proteoglycans, such as the chondroitin sulphates (3), and has not been found in any other mammalian glycoconjugate. Two more types of carbohydrate-protein linkages, commonly found in glycoproteins, have been recognized in proteoglycans, these being N-acetylglucosamine N-glycosidically linked to asparagine and N-acetylgalactosamine O-glycosidically linked to serine or threonine (3).

An interesting observation is that N-trifluoroacetylated chitosides have a higher affinity for wheat germ agglutinin than do N-acetyl chitosides (51), the binding constants being tenfold higher than those of the corresponding N-acetylated chitosides. These findings indicate that N-trifluoroacetylated oligosaccharides may be useful model compounds

when studying biological systems.

3.1.2 Uronic acids (Paper I)

Degradation of glucuronic acid and methyl α -glucopyranosiduronic acid by trifluoroacetolysis and analysis of the perethylated derivatives by g.l.c. resulted in the time curves shown in Fig. 2. A fast initial destruction of the hexuronic acids was followed by a much slower degradation,

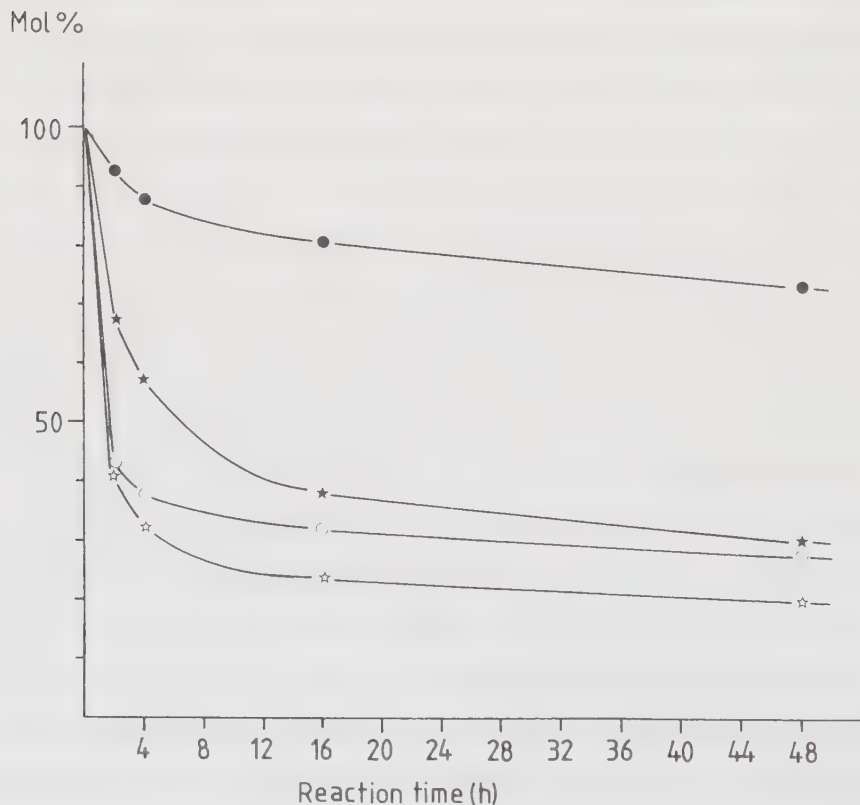
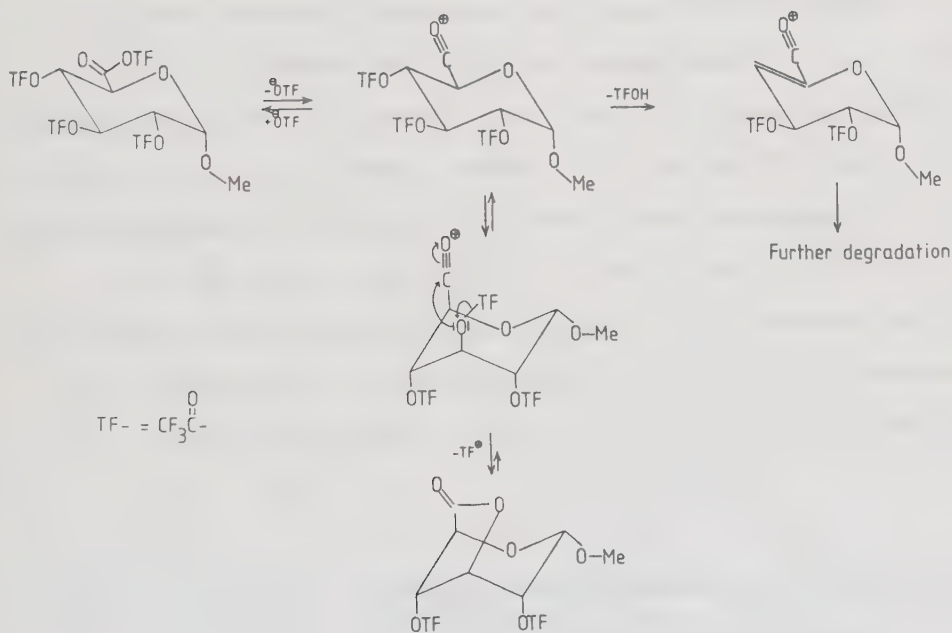


Fig. 2
Time curve of the degradation of glucuronic acid with 1:50 TFA/TFAA (○) or 1:1 TFA/TFAA (☆) and methyl α -glucuronic acid with 1:50 TFA/TFAA (●) or 1:1 TFA/TFAA (★).

which was interpreted as being due to the formation of an acylium ion in the first phase which caused elimination reactions in the pertrifluoroacetylated derivative. In the second phase 6,3-lactones were formed which prevent further degradation (Scheme 2).



Scheme 2

To prove these interpretations uronic acid derivatives incapable of forming 6,3-lactones and acylium ions were subjected to trifluoroacetolysis. Methyl 3-O-methyl- α -glycopyranosiduronic acid was completely degraded while methyl α -glucopyranosiduronic acid was recovered in 35% yield after 1:1 TFA/TFAA at 100° for 48 h. Under the same trifluoroacetolysis conditions methyl (methyl 4-O-methyl- α -gluco-

pyranoside) uronate was recovered in 85% yield.

This shows that elimination reactions cannot take place if an acylium ion cannot be formed and eliminations are prevented by formation of 6,3-lactones. Uronic acids with a free carboxylic acid group and incapable of forming 6,3-lactones are totally degraded and clearly demonstrate the role of a free carboxylic acid group in the elimination.

Glycosidic linkages of hexuronic acids are more resistant to acid hydrolysis than glycosidic linkages of corresponding hexoses (45). Trifluoroacetolysis on the other hand will degrade hexuronic acid residues rapidly if they possess a free carboxylic group and are incapable of forming 6,3-lactones. This can be useful in degradation of oligo- and polysaccharides containing hexuronic acid residues, when the hexuronic acids will be degraded specifically and neutral oligosaccharides can be isolated (see section 3.1.4)

3.1.3 Specific elimination of reducing

4-O-substituted hexoses (Paper I)

Aldonic acids (1-3) of isomaltotriose (α -Glc-(1-6)- α -Glc-(1-6)-Glc), laminaritriose (β -Glc-(1-3)- β -Glc-(1-3)-Glc) and maltotriose (α -Glc-(1-4)- α -Glc-(1-4)-Glc), respectively, were prepared by oxidation (52,53) and subjected to 1:50 TFA/TFAA at 100° for 48 h. Analysis by g.l.c.-m.s. after de-O-trifluoroacetylation, sodium borodeuteride reduction and permethylation revealed that the aldonic acids 1 and 2 could be recovered almost quantitatively as intact aldonic acids

(Table 1). The aldonic acid 3 was converted to the trifluoroacetylated enol of 3-deoxy-2-hexulosonic acid. Treatment of the de-0-trifluoroacetylated 3-deoxy-2-hexulosonic acid with mild base (0.05M NaOH) eliminated the 4-0-substituent from the reducing end. The resulting disaccharide (maltose) could be recovered quantitatively (Table 1).

Table 1. Treatment of aldonic acids, 1-3, with 1:50 TFA/TFAA and 1:50 TFA/TFAA with subsequent mild base

Compound	Recovery(%) ^a					
	TFA/TFAA (1:50)			TFA/TFAA (1:50) then base		
	A ^b	B	C	A	B	C
<u>1</u>	90	8	-	78	6	-
<u>2</u>	87	7	trace	57	10	7
<u>3</u>	-	70	28	-	-	101

^a Determined by g.l.c.-m.s as permethylated oligosaccharide alditols after de-0-trifluoroacetylation.

^b A aldonic acid and corresponding lactone,
 B hexulosonic acid and corresponding lactone,
 C disaccharide recovered after elimination of the reducing sugar.

Base labile linkages, as in aldonic acid 2, can be protected from peeling by adding sodium borohydride to the base (54).

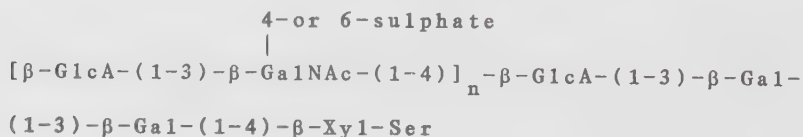
An application of this degradation mechanism is given in the following example. Glycosphingolipids frequently contain 3- or 4-0-substituted β -Gal-(1-4)-Glc carbohydrate chains attached to the sphingolipid. Trifluoroacetolysis of

these compounds releases the oligosaccharide chains. Oxidation of the released oligosaccharides to aldonic acids followed by a second trifluoroacetolysis step and subsequent mild base treatment will shorten the oligosaccharides by one residue (Glc) since this is 4-O-substituted. If the Gal residue is also 4-O-substituted the procedure can be repeated with concomitant release of the Gal unit. On the other hand any 3-O-substituent can be released by base treatment. By including sodium borohydride in the reaction mixture, the base susceptible linkages will be protected from degradation.

A combination of the above methods makes it possible to degrade an oligosaccharide chain sequentially from the reducing end. Degradation from the non-reducing end is possible by the use of specific exohydrolases. Thus, glycoconjugates can be degraded specifically to produce a whole range of oligosaccharides of potential biological interest.

3.1.4 Isolation of the linkage region oligosaccharides in chondroitin 4-sulphate (Paper II)

Chondroitin sulphate polysaccharides are O-glycosidically linked via xylose to serine in the protein core. The sulphate ester linkages in chondroitin sulphates are either at position 4 or 6 of the GalNAc residues (3,55). The carbohydrate structure of chondroitin sulphate is:



The oligosaccharides from the carbohydrate-protein linkage region in chondroitin 4-sulphate have been isolated by enzymatic degradation and the linkage region oligosaccharides have been isolated by acid hydrolysis and characterized by chemical methods (3,55). Degradation of proteoglycans by bacterial enzymes ('chondroitinases') probably occurs by the same mechanism as for alkaline β -elimination of uronic acids (56-58).

Trifluoroacetolysis of chondroitin 4-sulphate results in the degradation of the repeating disaccharide unit, $-3)-(SO_4^{\ominus}-4)-\beta$ -GalNAc-(1-4)- β -GlcA-(1-, by solvolysis of the glycosidic linkages. The GalNAc and GlcA residues are degraded due to insufficient protection of the glycosidic linkage by trifluoroacetyl groups.

The expected major product after trifluoroacetolysis of chondroitin 4-sulphate, β -Gal-(1-3)- β -Gal-(1-4)-Xyl (Table 2), is essentially stable due to the strong inductive effect

Table 2. Oligosaccharides obtained after trifluoroacetolysis of chondroitin 4-sulphate

Compound	yield(%) ^a
β -Gal-(1-4)-Xyl	12
β -Gal-(1-3)-Gal	23
β -Gal-(1-3)- β -Gal-(1-4)-Xyl	45
β -GlcA-(1-3)- β -Gal-(1-3)- β -Gal-(1-4)-Xyl	13

^a Determined by g.l.c.-m.s. as permethylated oligosaccharide alditols

exerted by the O-trifluoroacetyl groups (28,32). Analysis of the reduced and permethylated trisaccharide by g.l.c.-m.s. gave the mass spectrum shown in Fig 3.

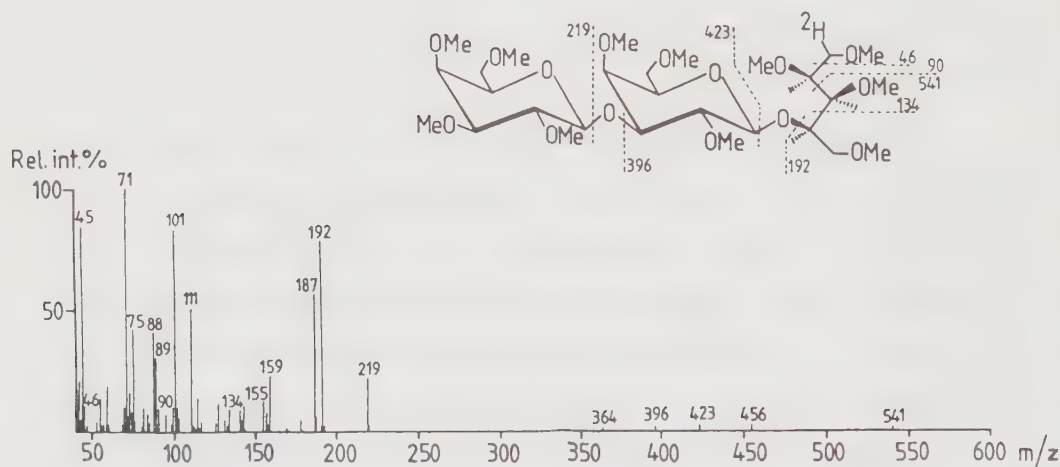


Fig. 3
Mass spectrum and some important primary fragments of the permethylated trisaccharide alditol from the linkage region of chondroitin 4-sulphate

However, if the glucuronic acid is prevented from degradation by formation of a 6,3-lactone (Scheme 2), the linkage region tetrasaccharide, β -GlcA-(1-3)- β -Gal-(1-3)- β -Gal-(1-4)-Xyl, can be isolated. After trifluoroacetolysis and reduction this tetrasaccharide was recognized by g.l.c.-m.s. as its permethylated derivative (Fig 4).

The disaccharide, β -Gal-(1-3)-Gal, was also isolated, possibly due to some degradation of the trisaccharide (28). The appearance of β -Gal-(1-4)-Xyl was not expected, and cannot be explained by degradation of the trisaccharide.

There is a possibility that linkage regions containing only one galactose unit, instead of the expected two, can be present in chondroitin 4-sulphate.

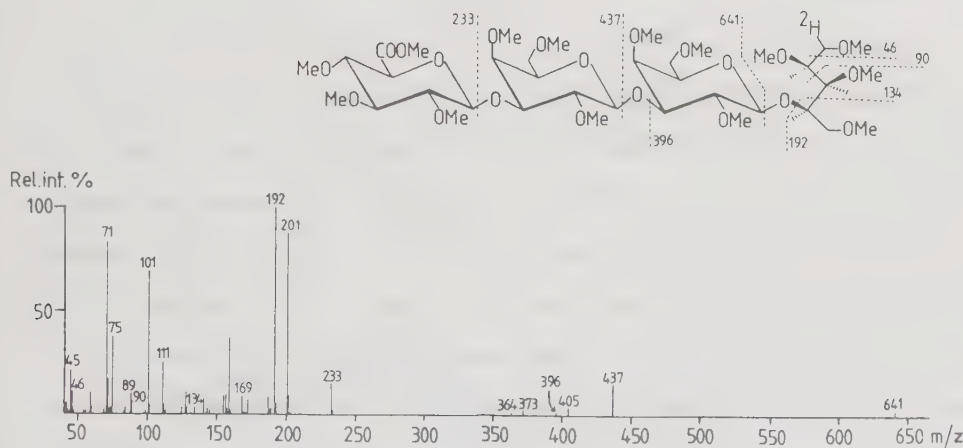


Fig. 4
Mass spectrum and some important primary fragments of the permethylated tetrasaccharide alditol from the linkage region of chondroitin 4-sulphate

A number of glycosyltransferases are responsible for the biosynthesis of the oligosaccharide chains in proteoglycans. Oligosaccharides isolated by trifluoroacetolysis from the carbohydrate-protein linkage region in proteoglycans can be used as substrates for testing the specificity of these transferases. Such oligosaccharides can be degraded specifically, for example, by elimination of the reducing 4-O-substituted xylose (see section 3.1.3) or by digestion with exohydrolases. Thus the number of related oligosaccha-

rides available for use in enzyme studies can be substantially increased.

3.2 Trifluoroacetolysis of glycosphingolipids

3.2.1 General

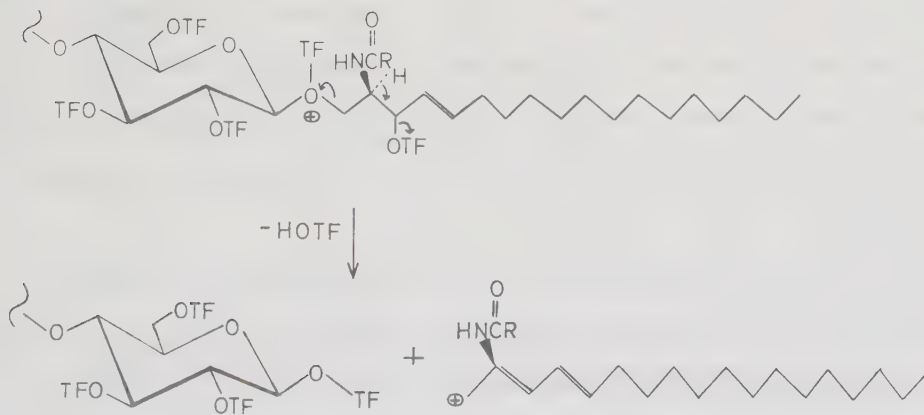
Glycosphingolipids can be divided into three groups according to their oligosaccharide chain, a) neutral glycosphingolipids, b) gangliosides having one or more sialic acid units and c) sulphatosphingolipids with sulphate ester groups as substituents of the carbohydrate chain (4,5). Glycosphingolipids consist of three basic units: a long-chain base, a fatty acid and a carbohydrate part. 4-Sphingenine is the most common long-chain base in mammalian glycosphingolipids, which when substituted with an amide-linked fatty acid of variable length forms a ceramide. The nomenclature of glycosphingolipids is complex, but subdivisions are based on the oligosaccharide structure (4).

Analyses of carbohydrate chains in glycosphingolipids have usually been achieved after purification using tedious isolation procedures followed by structural analysis of the purified component (5,59). Isolation of pure glycosphingolipids in sufficient quantities for characterization can be difficult to accomplish because of the small quantities present in biological tissues. Recent improvements in methodology for the isolation of specific glycosphingolipids from complex mixtures have been made, such as specific

antibody binding on thin-layer plates (60,61).

3.2.2 Trifluoroacetytolysis of neutral glycosphingolipids (Paper III)

Release of oligosaccharides from glycosphingolipids is achieved by trifluoroacetytolysis and probably proceeds via elimination of an O-trifluoroacetyl group in the sphingosine moiety, yielding an allyl cation (Scheme 3) (62). This cleavage mechanism requires an unsaturated sphingosine base (30).



Scheme 3

An alternative cleavage mechanism is an electrophilic addition of a trifluoroacetylum ion to the double bond with subsequent elimination of trifluoroacetic acid.

Rate studies of the cleavage of the glycosidic bond in galactosylceramide showed that a 21 and 27 h reaction time is needed to accomplish complete cleavage in 1:1 TFA/TFAA and 1:50 TFA/TFAA, respectively. Longer reaction times were

needed to transamidate completely any N-acetyl groups present in glycosphingolipids.

When glycosphingolipids, not containing more than four sugar units in the oligosaccharide chain, were subjected to various trifluoroacetolysis conditions quantitative recovery of the oligosaccharides could be obtained.

Degradation of the oligosaccharide chain, α -GalNAc-(1-3)- β -GalNAc-(1-3)- α -Gal-(1-4)- β -Gal-(1-4)-Glc, of the Forssman antigen could be seen in trifluoroacetolysis when higher trifluoroacetic acid concentrations were used. Using milder TFA/TFAA conditions (1:100) the oligosaccharide chain could be recovered in 83% yield, in contrast to the findings of Nilsson and Zopf who found only degradation products (30). The pentasaccharide chain of the Forssman antigen was isolated after trifluoroacetolysis and the identity of the pentasaccharide was verified by sugar and methylation analyses (63,64).

The direct probe mass spectrum further confirmed the Forssman antigen pentasaccharide structure (Fig. 5). From the non-reducing end the fragments m/z 709 ($cabA_1$), m/z 505 (baA_1), m/z 260 (aA_1) and m/z 228 (aA_2) (elimination of methanol from m/z 260 and elimination of 3,4,6-tri-O-methyl-hexN(Me)Ac from m/z 505) of the A-series could be seen (20,65). Furthermore the fragments m/z 236 and 296 from the reducing end (J-series) show a penta-O-methyl hexitol.

Support for the identity of the pentasaccharide was obtained by proton n.m.r. spectroscopy. The anomeric configuration indicated by the n.m.r. spectrum was as expected

for the oligosaccharide and the number of N-acetyl groups was correct.

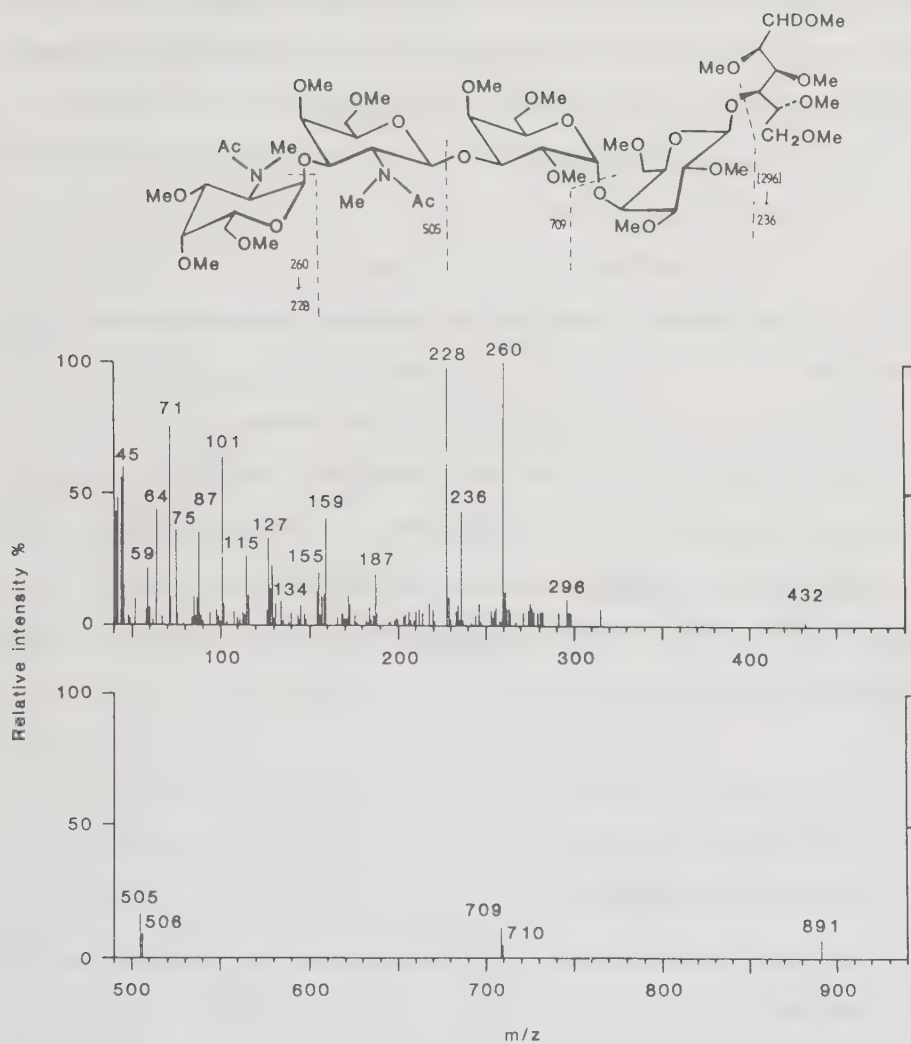


Fig. 5

Mass spectrum and some important primary fragments of the Forssman antigen pentasaccharide as its permethylated alditol.

The lower recovery of oligosaccharide chains from some of the glycosphingolipids can be explained by the presence of ceramides with saturated long-chain bases which would prevent cleavage of the glycosidic bond to the ceramide.

Trifluoroacetolysis of glycosphingolipid mixtures will release the oligosaccharides which are linked to an unsaturated sphingosine base. Transamidation will cleave off the fatty acid chains and the liberated oligosaccharides can be separated from the glycosphingolipids with a saturated sphingosine base by gel chromatography after trifluoroacetolysis (66). This method can be used to isolate those glycosphingolipids having saturated sphingosine bases. This possibility may well be useful in studying glycosphingolipid metabolism.

Isolation of oligosaccharides from gangliosides are difficult to accomplish without destroying the sialic acid residues. By using mild trifluoroacetolysis conditions sialic acid-containing oligosaccharides from gangliosides can be recovered in relative good yield (60-65% with 1:100 TFA/TFAA at 100° for 48 h) (67).

4. STRUCTURAL STUDIES

4.1 General

The combination of trifluoroacetolysis and mass spectrometry is useful in structural studies of glycoconjugates. Oligosaccharide chains have been released from glycoconjugates by trifluoroacetolysis and analysed by m.s. as their permethylated oligosaccharide alditols, both as N-trifluoroacetyl derivatives (30,42) and after reconstitu-

tion to N-acetyl derivatives (29). Furthermore, the oligosaccharides of human antithrombin (27), human thrombin (68), blood group active glycoproteins (69) and glycolipid hybridoma cell lines (70) have been characterized by this method. By using specific antibodies a novel glycosphingolipid was isolated and characterized by trifluoroacetolysis-m.s. (71).

4.2 Structural studies on the O-linked carbohydrate chains in glucoamylase G1 (Paper IV)

Glucoamylases (EC 3.2.1.3) are exohydrolases produced on a large scale by various fungi and are of industrial importance in catalyzing the release of glucose from starch and related oligo- and polysaccharides (72-75). One application is in the brewing industry where they are used to produce low calorie beer.

It has been shown that the O-linked carbohydrate chains of glucoamylase G1 are located in a 70 amino acid fragment out of the total of 616 amino acids present (76). The glycosylated segment carries about 35 oligosaccharide units (76).

The highly glycosylated glycopeptide, Tca2-1, consisting of the 70 amino acid segment was prepared from glucoamylase G1 (76,77). Sugar analysis of Tca2-1 showed a carbohydrate content of 50% (wt/wt) and the dominating monosaccharides were Man and Glc in the molar ratio 6:1. The carbohydrate chains released by trifluoroacetolysis from glycopeptide Tca2-1 showed the same monosaccharide composition as in the intact glycopeptide. Methylation analysis of the glycopeptide

Tca2-1 gave inter alia the structural elements 6-O-substituted Man, 2-O-substituted Man, 2,6-di-O-substituted Man, and non-reducing terminal Man and Glc. When the oligosaccharides liberated by trifluoroacetolysis from the tryptic fragment Tca2-1 were subjected to methylation analysis they revealed hexa-O-methyl-Man. This shows that single mannosyl residues are common in glucoamylase G1.

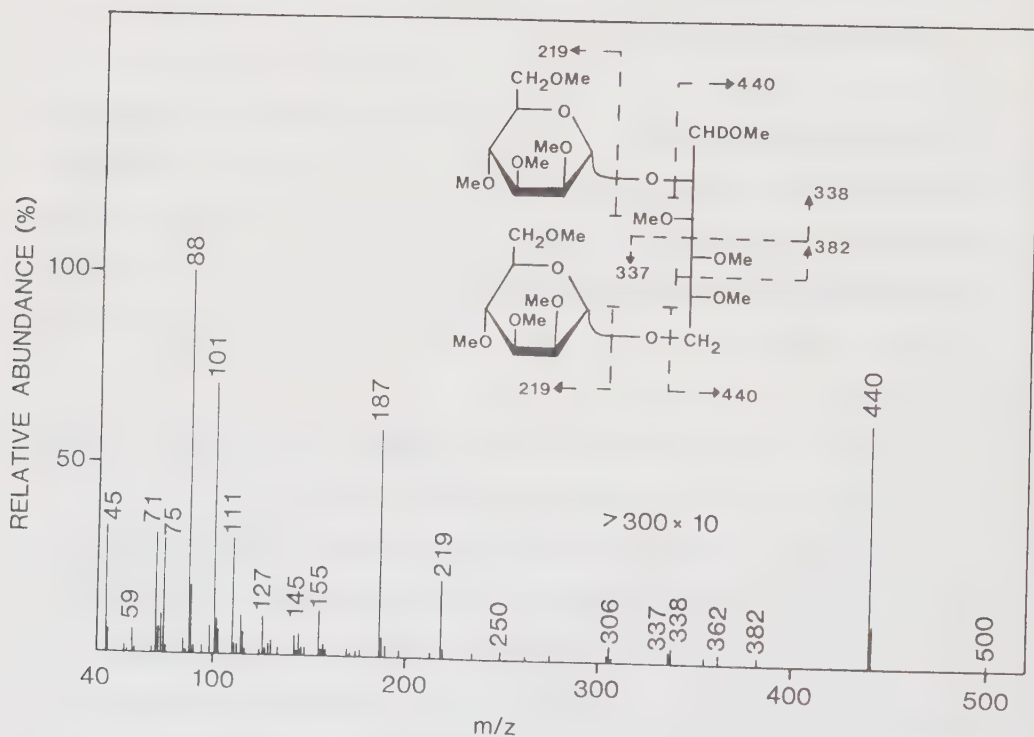
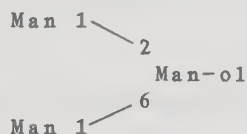


Fig. 6
Mass spectrum of the permethylated trisaccharide alditol obtained from glycopeptide Tca2-1 by trifluoroacetolysis and reduction.

Trifluoroacetolysis of the glycopeptide released oligosaccharides which were analysed by g.l.c.-m.s. as permethylated alditols. A branched trisaccharide alditol was identi-

fied. The mass spectrum of the trisaccharide (Fig. 6) showed the presence of ions for a non-reducing terminal hexose (m/z 219, m/z 187 and m/z 155). The absence of ions for a non-reducing disaccharide sequence showed that the alditol must be substituted with two hexosyl residues. Substitution in the 2- and 6-position of the alditol was indicated by ions from cleavages within the alditol, m/z 337, m/z 338 and m/z 306 (elimination of methanol from m/z 338). The 2,6-substitution of the alditol was further confirmed by the methylation analysis of the glycopeptide Tca2-1. The branched trisaccharide alditol must therefore have a branching pattern unusual for glycoprotein oligosaccharides:



This structure can be differentiated by m.s. from a 3,6-di-O-substituted Man which is common in glycoproteins. The ion m/z 338 is formed by cleavage between carbon 3 and 4 in the alditol chain (see Fig. 6) and the cleavage preferentially takes place between carbons substituted with methoxyl groups (20). In a 3,6-di-O-substituted Hex-ol it is unlikely that this unfavoured cleavage takes place when the charge would be located on the glycosidic oxygen (26). This is further indicated by the absence of elimination of methanol to give m/z 306.

Other oligosaccharides released from the glycopeptide Tca2-1 by trifluoroacetolysis, are shown in Table 3.

Table 3. Oligosaccharides from glycopeptide Tca2-1 released by trifluoroacetolysis.

Compound	moles/mole glycopeptide
Man	5
Man α 1-2 Man	5
Hex 1-6 Hex	2
Man α 1-6 Man	<1
Man α 1 $\begin{array}{l} \diagdown \\ 2 \\ \text{Man} \end{array}$	11
Man α 1 $\begin{array}{l} \diagdown \\ 6 \end{array}$	

Hex-(1-6)-Hex in Table 3 could not be positively identified, but the non-reducing terminal is probably Glc. It is possible to differentiate non-reducing gluco- from the manno- and galacto-configurations by m.s. (78). Further evidence was provided by the relative retention time on a SE-30 vitreous silica capillary column which was the same as that of α -Glc-(1-6)-Glc and α -Glc-(1-6)-Man.

By α -mannosidase digestion and chromium trioxide oxidation (79), of the oligosaccharides, it was shown that the majority of the glycosidic linkages have the α -configuration.

When glucoamylase G1 or glycopeptide Tca2-1 was digested with α -mannosidase a precipitate appeared. A precipitate could also be seen when glucoamylase G1 was treated with hydrogen fluoride to remove the carbohydrate

chains. The protein itself is less soluble without carbohydrate chains which may explain the slightly lower release of Man under digestion of the glycopeptide Tca2-1 with α -mannosidase.

Glucoamylase G1 from Aspergillus niger is an unusual glycoprotein, since it carries a large number of short O-glycosidically linked carbohydrate chains, consisting mainly of mannose (80,81). Glucoamylase G1 contains 19% carbohydrate (wt/wt) and besides the highly glycosylated region (Tca2-1) the glycoprotein also contains two N-glycosylated asparagine residues (82). Glucoamylase G1 has a capacity to digest raw starch in contrast to glucoamylase G2, which is glycosylated to the same degree but is lacking a C-terminal amino acid sequence (77).

The biological function of the carbohydrate moities of glucoamylase is not known. It has been speculated that the glycosylated region is required for the adherence to the substrate, but glucoamylase G2 is glycosylated to the same extent as glucoamylase G1 and cannot adsorb to and digest raw starch. However, the oligosaccharides are polar groups which may keep the glucoamylase in a certain conformation so that the active site may be exposed. The high density of oligosaccharide chains in the 70 amino acid fragment may give this specific conformation to glucoamylase. Another simpler explanation is that the carbohydrates keep the glucoamylase in solution which is supported by the α -mannosidase and HF-treatments. For other glycoproteins the carbohydrate function is unknown but it seems unlikely that the sophisticated machinery of glycosyltransferases is working in vain.

4.3 Oligosaccharides mediating agglutination of sheep erythrocytes (Paper V)

Pathogenicity is the ability of microorganisms to cause disease. In many cases the adhesion of pathogens to host tissues has been shown to be an important factor in causing infections (8,83). The adherence mechanism is dependent on physico-chemical factors (83,84) and the presence of specific carbohydrate receptors (7,8,85).

Urinary tract infections are very common and Staphylococcus saprophyticus is one of the causative agents, especially in young women (86-88). In most cases the upper urinary tract is involved in infections caused by S. saprophyticus and can result in a reduced concentration capacity of the kidney (89). In humans, S. saprophyticus is exclusively localised to the urogenital tract.

S. saprophyticus has the ability to agglutinate sheep erythrocytes in contrast to other coagulase-negative staphylococcal species, such as S. aureus (90). The inhibition of this phenomenon by oligosaccharides has been investigated.

Glycosphingolipids were prepared from sheep erythrocyte membranes by conventional methods. After trifluoroacetolysis of the glycosphingolipids (1:50, 100⁰, 48 h), the liberated oligosaccharide mixtures were tested for their ability to inhibit the haemagglutination of sheep erythrocytes by S. saprophyticus. Oligosaccharides from two glycosphingolipid fractions showed inhibitory activity and the glycosphingolipid fractions were rechromatographed on Silica Gel columns

and further purified by preparative thin-layer chromatography. After release of the oligosaccharides by trifluoroacetylysis, fraction 1:III and 3:III showed high inhibitory activity (Table 4). Sugar and methylation analysis of fraction 1:III indicated that this fraction consisted of the Forssman antigen oligosaccharide, α -GalNAc-(1-3)- β -GalNAc-(1-3)- α -Gal-(1-4)- β -Gal-(1-4)-Glc, which was confirmed by direct inlet m.s. and n.m.r. (see section 3.2.2). Sugar analysis of fraction 3:III revealed the monosaccharides Gal, Glc and GlcNAc, and methylation analysis showed inter alia non-reducing terminal Gal, 4-0-substituted Gal, 3-0-substituted Gal, 4-0-substituted GlcNAc and 3,6-di-0-substituted Gal. A non-reducing terminal disaccharide sequence, Hex-(1-4)-HexNAc, was shown to be present in fraction 3:III by the formation of Hex-(1-4)-2,5-anhydro-Hex by nitrous acid deamination (91), and it was concluded that the structure was β -Gal-(1-4)-GlcNAc (lactosamine).

A number of oligosaccharides were tested for their ability to inhibit the haemagglutination of sheep erythrocytes by S. staphylococcus (Table 4). Those which contained a non-reducing terminal lactosamine showed inhibitory activity at low concentrations, whilst oligosaccharides lacking this non-reducing terminal sequence were inactive at 10 times higher concentrations.

The lactosamine sequence is very common in glycoproteins, but frequently the Gal residue is substituted by a sialic acid unit either in the 3- or 6-position. Native glycoproteins containing galactose units substituted by sialic acids were inactive whereas the corresponding de-

Table 4. Haemagglutination-inhibition test. Carbohydrate structures as inhibitors of the haemagglutination of sheep erythrocytes by Staphylococcus saprophyticus

Inhibitor	Minimum inhibitory concentration µg/ml
β-Gal-(1-4)-GlcNAc	300
β-Gal-(1-4)-β-GlcNAc-(1-0)-Me	250
β-Gal-(1-4)-β-GlcNAc-(1-3)-β-Gal-(1-4)-Glc	250
β-Gal-(1-4)-β-GlcNAc-(1-2)-α-Man-(1-6)-β-Man-(1-4)-GlcNAc	250
β-Gal-(1-4)-β-GlcNAc-(1-2)-α-Man-(1-6)	200
β-Gal-(1-4)-β-GlcNAc-(1-2)-α-Man-(1-3)	200
β-Gal-(1-4)-β-GlcNAc-(1-2)-α-Man-(1-6)	
β-Gal-(1-4)-β-GlcNAc-(1-2)-α-Man-(1-3)	200
β-Gal-(1-3)-β-GlcNAc-(1-4)-β-Gal-(1-4)-Glc	
β-GalNAc-(1-3)-α-Gal-(1-4)-β-Gal-(1-4)-Glc	>2500
α-Gal-(1-4)-β-Gal-(1-4)-Glc	>2500
β-Gal-(1-4)-Gal	>2500
β-Gal-(1-4)-Glc	>2500
Antithrombin III (human)	>2500
Asialoantithrombin III (human)	250
Transferrin (human)	>2500
Asialotransferrin (human)	>2500
Fetuin	250
Asialofetuin	>2500
α-NeuNAc-(2-6)-β-Gal-(1-4)-Glc	125
α-NeuNAc-(2-3)-β-Gal-(1-4)-Glc	>2500
Fraction 1:III	>2500
Fraction 3:III	300
	150

sialylated glycoproteins showed high inhibitory activity (Table 4).

The binding of carbohydrate structures to combining sites is probably caused by specific hydrophobic interactions (23). Conformational analysis of structures with non-reducing terminal lactosamine sequences has indicated the structure depicted in Fig. 7 (23). This conformation is due to a

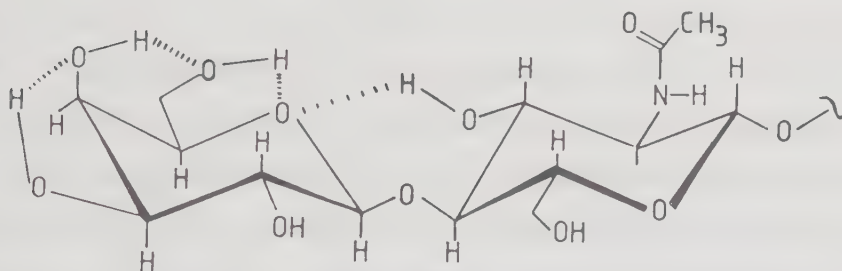


Fig. 7
Preferred conformation of β -Gal-(1-4)- β -GlcNAc-(1-

hydrogen-bonding network involving the hydroxyl groups at C-3, C-4 and C-6 of the galactose unit (Fig. 7). The conformation shown in Fig. 7 is most interesting, since one 'side' of the structure is hydrophobic, especially if the hydrogen bond between the hydroxyl group of C-3 in the GlcNAc residue and the ring oxygen of the Gal unit are also taken into account (23). Thus, carbohydrate chains which are normally considered to be very hydrophilic because of the abundance of hydroxyl groups, can have hydrophobic domains which are suitable for attachment to receptor sites.

The observations that fraction 1:III, consisting of the Forssman antigen pentasaccharide, showed good inhibitory ac-

tivity cannot readily be explained. It is possible that the Forssman antigen pentasaccharide possesses a hydrophobic surface similar to the lactosamine structure and will fit into the hydrophobic cleft of the combining site. The receptor site has very specific requirements, since changing the linkages (1-4 to 1-3) or the monosaccharide units (GlcNAc to Glc) abolishes the inhibition (Table 4).

Earlier investigations have shown that the S. saprophyticus haemagglutinin is heat sensitive and susceptible to proteolytic enzymes (89) and thus is probably a membrane protein. It is likely that the specific ability of S. saprophyticus to adhere to urothelial cells is caused by the same membrane protein that causes the haemagglutination, since the presence of β -Gal-(1-4)-GlcNAc has been shown in human urinary bladder epithelium (92).

5. SUMMARY

Trifluoroacetolysis is a versatile reaction for use in the isolation and characterization of oligosaccharide chains in glycoconjugates. Trifluoroacetolysis is performed in various mixtures of trifluoroacetic acid and trifluoroacetic anhydride at elevated temperatures. Oligosaccharides are stable, under trifluoroacetolysis conditions, due to the strong inductive effect exerted by the O-trifluoroacetyl groups introduced into the sugar moieties.

In this dissertation further applications of the trifluoroacetolysis reaction have been discussed. Degradation of hexuronic acids by trifluoroacetolysis is initiated by acylium ion formation in the carboxylic acid group. This acylium ion is in equilibrium with a 6,3-lactone, which prevents further elimination reactions. By utilizing this phenomenon it was possible to develop a method for the specific elimination of reducing 4-O-substituents in hexose residues. Appropriate oligosaccharides are oxidized to aldonic acids which after trifluoroacetolysis and subsequent base treatment eliminate the substituent at position 4 of the reducing end sugar.

Trifluoroacetolysis of chondroitin 4-sulphate enabled the isolation of oligosaccharides from the carbohydrate-protein linkage region tetrasaccharide, β -GlcA-(1-3)- β -Gal-(1-3)- β -Gal-(1-4)-Xyl. The trisaccharide, β -Gal-(1-3)- β -Gal-(1-4)-Xyl was the major component isolated but the complete linkage region tetrasaccharide could also be isolated due to

lactone formation of the GlcA residue, which prevents degradation of the hexuronic acid.

Most of the glycosphingolipids tested yielded intact oligosaccharides after trifluoroacetolysis. The exception was the Forssman antigen, a more complex glycosphingolipid, in which degradation of the oligosaccharide chain took place under severe trifluoroacetolysis conditions. Using milder conditions the Forssman pentasaccharide was recovered in good yield.

After liberation of the O-glycosidically-linked oligosaccharide chains in glucoamylase G1 by trifluoroacetolysis, the oligosaccharides were characterized. An unusual branched trisaccharide, α -Man-(1-2)-[α -Man-(1-6)-]-Man, was found.

Trifluoroacetolysis of glycosphingolipids from sheep erythrocyte membranes liberated the oligosaccharide chains. The released oligosaccharides were tested as inhibitors of the haemagglutination of sheep erythrocytes by Staphylococcus saprophyticus. Oligosaccharides containing β -Gal-(1-4)-GlcNAc as a terminal non-reducing disaccharide sequence were good inhibitors of the haemagglutination.

6. ACKNOWLEDGMENTS

This work could not have been completed without the generous advice given by colleagues at the Department of Carbohydrate Chemistry, the Department of Clinical Chemistry and the Department of Medical Microbiology, University of Lund.

My sincere gratitude is extended to:-

Prof Sigfrid Svensson, my supervisor, for his guidance and unflinching interest when introducing me to the field of carbohydrate chemistry.

Drs Bo Nilsson and Frank Lindh for their comments and criticism of this work.

Prof Per Arne Öckerman for putting facilities at my disposal.

Dr Alan Chester for excellent advice on writing in English.

Karin Erlansson, Lisbeth Palm-Svensson, Ewa Persson and Stefan Strömberg for skilful technical assistance, Göran Nilsson for many interesting discussions and Katarina Danielsson for secretarial help.

This work was supported by grants from the Swedish Medical Research Council (16X-4509).

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THE REACTIVITY OF URONIC AND ALDONIC ACID DERIVATIVES TOWARDS
TRIFLUOROACETOLYSIS. A NEW METHOD FOR SPECIFIC ELIMINATION OF
REDUCING 4-O-SUBSTITUTED HEXOSE RESIDUES

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ABSTRACT

Trifluoroacetolysis of D-glucuronic acid and methyl α -D-glucopyranosiduronic acid resulted in an initial phase of degradation followed by stabilization of the compounds as their 6,3-lactones. The methyl ester of methyl 4-O-methyl- α -D-glucopyranosiduronic acid was largely stable towards trifluoroacetolysis. Aldonic acids substituted at position 3 or 6 were stable towards trifluoroacetolysis due to formation of γ -lactones. Aldonic acids substituted at position 4 and incapable of forming γ -lactones, were converted into the trifluoroacetylated enol of 3-deoxy-2-hexulosonic acid. Treatment of the 3-deoxy-2-hexulosonic acid with mild base eliminated the substituent at position 4.

INTRODUCTION

Trifluoroacetolysis is a versatile reaction in studies of glycoconjugates and has been used for the N-deacetylation of 2-acetamido-2-deoxy sugars¹, the isolation of N- or O-glycosidically linked carbohydrates from glycoproteins²⁻⁴, and to release carbohydrates from glycosphingolipids^{5,6}. The usefulness of the trifluoroacetolysis reaction depends on the electron attracting character of the O-trifluoroacetyl groups introduced into the sugar moieties which dramatically retards the rates of solvolysis of glycosidic bonds⁷⁻¹¹. We now report on the reactivity of uronic and aldonic acid derivatives under trifluoroacetolysis conditions.

MATERIALS AND METHODS

Methyl α -D-glucopyranosiduronic acid was prepared by catalytic oxidation (Pt/O_2) of methyl α -D-glucopyranoside¹². Methyl 3-O-methyl- α -D-glucopyranoside was prepared by Purdie methylation of methyl 4,6-O-benzylidene- α -D-glucopyranoside¹³ and debenzylidenation with trifluoroacetic acid - trifluoroacetic anhydride (1:50, 50°, 1 h). Catalytic oxidation of the primary hydroxyl group with freshly reduced Adams catalyst¹⁴ and oxygen then gave methyl 3-O-methyl- α -D-glucopyranosiduronic acid¹⁵. Methyl (methyl 4-O-methyl- α -D-glucopyranoside) uronate and maltotriose were commercial products, isomaltotriose was obtained by partial acid hydrolysis of dextran¹⁶, and laminari-

triose by partial acid hydrolysis of laminaran¹⁷. The aldonic acids of the trisaccharides were obtained by oxidation with hypiodite^{18,19}.

Analytical methods. - G.l.c. was carried out on a Perkin-Elmer 3920 gas chromatograph equipped with a flame ionisation detector and a SE-30 W.C.O.T. glass capillary column (25 m x 0.25 mm) at 140-180° (for perethylated uronic acid derivatives) and at 180-330° (for permethylated aldonic acid derivatives). G.l.c.-m.s. was performed on a Varian MAT 311A instrument fitted with the appropriate column. The spectra were recorded at 70 eV, with an ionisation current of 3 mA and an ion source temperature of 120°. The spectra were processed by an on-line computer system (Spectrosystem 100, Varian MAT)

Trifluoroacetolysis. - Compounds were treated with mixtures of trifluoroacetic acid and trifluoroacetic anhydride in proportions varying from 1:1 to 1:50 (2 mL/mg of oligosaccharide) at 100° for 48 h (caution, corrosive mixture under pressure). Each cooled mixture was concentrated to dryness and a solution of the residue in methanol (5 mL) was again concentrated to dryness. The residue was dissolved in glacial acetic acid (5 mL), water (5 mL) was added, and after storage for 30 min at 100°, the solution was concentrated to dryness.

Base treatment. - The products of trifluoroacetolysis were stirred with 0.05M NaOH (5 mL) overnight at room temperature. Each mixture was neutralised with Dowex 50 (H⁺) resin, concentrated to dryness, and the residue was reduced with NaBD₄. The reduction was stopped with Dowex 50 (H⁺) resin and the mixture was concentrated to dryness

after adding methanol. The product was permethylated²⁰ and analysed by g.l.c.²¹ and m.s.²².

RESULTS AND DISCUSSION

Treatment of D-glucuronic acid (D-GlcpA) and methyl α -D-glucopyranosiduronic acid (Me α -D-GlcpA) with a mixture of trifluoroacetic acid (TFA) and trifluoroacetic anhydride (TFAA) in various proportions at 100° gave, after permethylation of the product and analysis by g.l.c., the time curves shown in Fig. 1. Initial fast reaction was followed by

Mol %

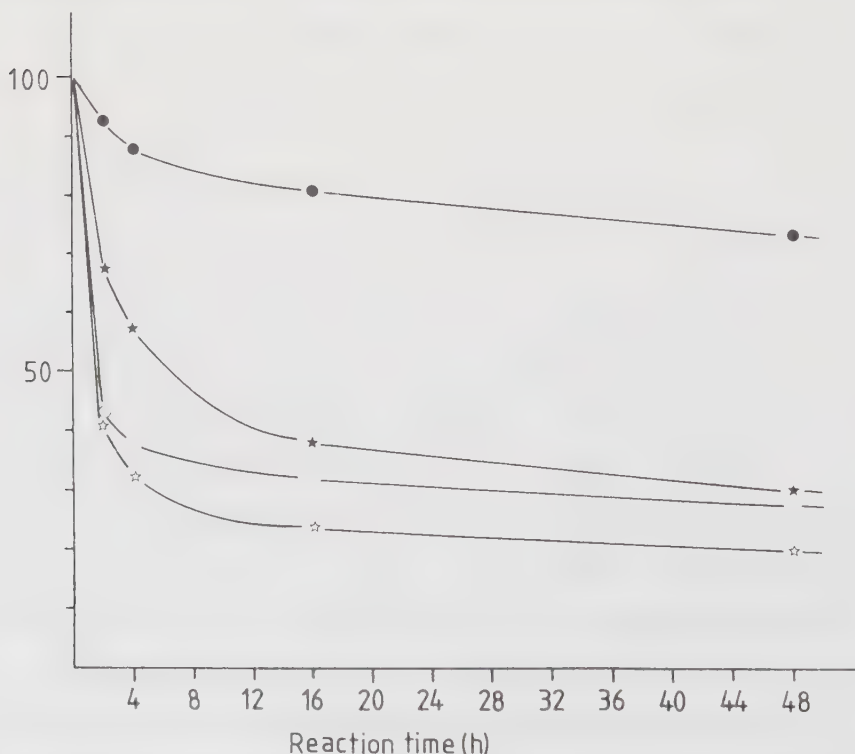
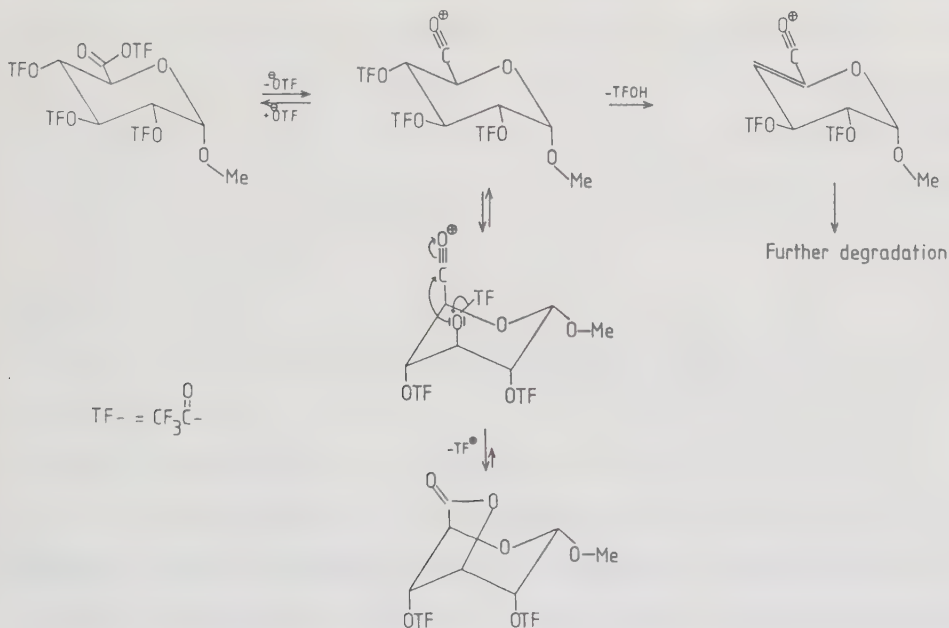


Fig. 1

Time curve of the degradation of D-glucuronic acid with 1:50 TFA/TFAA (○) and 1:1 TFA/TFAA (☆) and methyl α -D-glucopyranosiduronic acid with 1:50 TFA/TFAA (●) and 1:1 TFA/TFAA (★).

slower degradation of D-GlcpA and Me α -D-GlcpA. The first phase reflects the degradation of the trifluoroacetylated derivatives via eliminations from an acylium ion and the second phase reflects the formation of 6,3-lactones which are degraded more slowly (Scheme 1). The 6,3-lactones are in



Scheme 1

equilibrium with an acylium ion from which elimination can take place, but the formation of lactones is favoured and they are stable towards trifluoroacetolysis.

In order to verify this interpretation, methyl 3-O-methyl- α -D-glucopyranosiduronic acid (Me 3- α -D-GlcpA) and the methyl ester of methyl 4-O-methyl- α -D-glucopyranosiduronic acid [Me(Me 4- α -D-Glcp)uronate] were subjected to trifluoroacetolysis. In 1:1 TFA/TFAA 35% of Me α -D-GlcpA was recovered whereas Me 3- α -D-GlcpA was completely degraded since it cannot form a 6,3-lactone (Table I).

Table I

Treatment of uronic acids with TFA/TFAA at 100°C for 48 h

TFA/TFAA	Recovery (%) ^a			
	1:1	1:4	1:15	1:50
Me α - <u>D</u> -Glc <u>p</u> A	35	58	65	77
Me 3- α - <u>D</u> -Glc <u>p</u> A	1	7	25	56
Me (Me 4- α - <u>D</u> -Glc <u>p</u>) uronate	85	85	90	93

^a Determined by g.l.c.-m.s after de-O-trifluoroacetylation and perethylation.

When the carboxylic acid group is esterified no acylium ion can be formed and no elimination can take place. The glycosidic bond is stabilized by the inductive effect of the O-trifluoroacetyl groups. The recovery of Me (Me 4- α -D-Glcp) uronate after treatment with 1:1 TFA/TFAA is slightly lower than that of Me 4- α -D-Glcp⁸ due to the absence of a 6-O-trifluoroacetyl group in the former compound but is approximately the same as that of Me 4,6- α -D-Glcp⁸. When the carboxylic acid is not esterified an acylium ion can be formed and elimination can occur. However, elimination will be prevented if a 6,3-lactone can be formed.

The role of a free carboxylic acid and the inability to form a 6,3-lactone is reflected by the recoveries of Me 3- α -D-GlcpA (1%, Table I) and Me 3- α -D-Glcp (97%)⁸ after treatment with 1:1 TFA/TFAA. A decrease in the proportion of TFA in the trifluoroacetylolysis mixture resulted in higher

recoveries of the D-GlcpA derivatives (Table 1) and was most marked for those compounds (Me α -D-GlcpA and Me 3- α -D-GlcpA) which contained a free carboxylic acid group, possibly because the formation of acylium ion was progressively disfavoured.

The above results prompted an investigation of the possibility of specific elimination of a 4-O-substituted hexose residue at the reducing end of appropriate oligosaccharides. The aldonic acids (1-3) of isomaltotriose, laminaritriose and maltotriose were treated severally with

Table II

Treatment of aldonic acids, 1-3^a, with 1:50 TFA/TFAA and 1:50 TFA/TFAA and subsequent base (0.05M NaOH)

Compound	Recovery(%) ^b					
	TFA/TFAA (1:50)			TFA/TFAA (1:50) then base		
	A ^c	B	C	A	B	C
<u>1</u>	90	8	-	78	6	-
<u>2</u>	87	7	trace	57	10	7
<u>3</u>	-	70	28	-	-	101

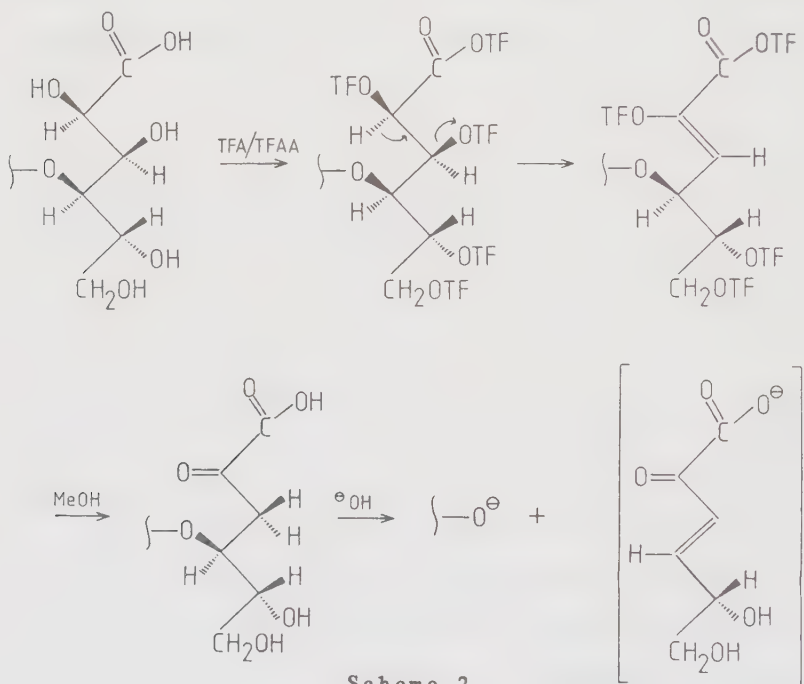
^a Derived from isomaltotriose, laminaritriose, and maltotriose respectively.

^b Determined by g.l.c.-m.s as permethylated oligosaccharide alditols after de-O-trifluoroacetylation.

Key:

- ^c A aldonic acid and corresponding lactone,
 B hexulosonic acid and corresponding lactone,
 C disaccharide eliminated from the reducing end.

1:50 TFA/TFAA for 48 h at 100° together with gentiobiose as an inert internal standard. The reaction products were de-Q-trifluoroacetylated, reduced with sodium borodeuteride, permethylated, and subjected to g.l.c.-m.s. As can be seen from the data in Table II, 1 and 2 were recovered almost completely (as mixtures of free aldonic acid or lactone) whereas 3 was converted into the trifluoroacetylated enol of 3-deoxy-2-hexulosonic acid (Scheme 2). Treatment of the



Scheme 2

3-deoxy-2-hexulosonic acid, after de-Q-trifluoroacetylation, with mild base eliminated the substituent at position 4 (Scheme 2) and gave a quantitative yield of the resulting disaccharide (Table II). Treatment of the aldonic acids 1 and 2 with mild base did not release the corresponding disaccharide due to the formation of γ -lactones (Table II), which were stable towards trifluoroacetolysis. The lower recovery

of 2 after treatment with base is due to a peeling reaction from the reducing end.

Thus, after oxidation of the reducing terminus of an oligosaccharide to an aldonic acid 4-O-substituents can be eliminated specifically therefrom by trifluoroacetolysis followed by treatment with base. Oligosaccharides with base-labile linkages released in this process can be protected from a peeling reaction by including sodium borohydride in the base²³.

ACKNOWLEDGMENTS

The authors thank Mr S. Strömberg for skilful technical assistance and the Swedish Medical Research Council (3X-4956) and the Medical Faculty, University of Lund, for financial support.

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DEGRADATION OF CHONDROITIN 4-SULPHATE BY TRIFLUOROACETOLYSIS.
ISOLATION OF OLIGOSACCHARIDES FROM THE CARBOHYDRATE-PROTEIN
LINKAGE REGION

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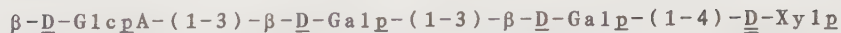
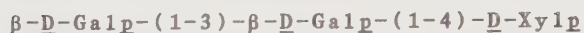
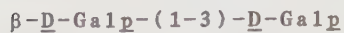
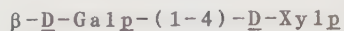
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ABSTRACT

Oligosaccharides from the linkage region tetrasaccharide, β -D-Glc_pA-(1-3)- β -D-Gal_p-(1-3)- β -D-Gal_p-(1-4)-D-Xyl_p, of chondroitin 4-sulphate were isolated after trifluoroacetolysis. The oligosaccharides were purified by ion exchange chromatography and paper chromatography and analysed by sugar analysis, methylation analysis and gas-liquid chromatography-mass spectrometry. The recovery of linkage region oligosaccharides was about 45% (wt/wt) after trifluoroacetolysis calculated according to the D-xylose present in the chondroitin 4-sulphate preparation. The following

structures were identified,



INTRODUCTION

In mammalian proteoglycans most polysaccharide species are linked to the core proteins by an O-glycosidic linkage between D-xylose and serine¹. The D-xylose residue is part of a distinct linkage region tetrasaccharide of the following structure: $\beta\text{-}\underline{\underline{\text{D}}}\text{-GlcAp}-(1-3)\text{-}\beta\text{-}\underline{\underline{\text{D}}}\text{-Galp}-(1-3)\text{-}\beta\text{-}\underline{\underline{\text{D}}}\text{-Galp}-(1-4)\text{-}\underline{\underline{\text{D}}}\text{-Xylp}$. For the chondroitin sulphate proteoglycans of cartilage, this structure was established by enzymatic degradation of the proteoglycans to glycopeptides followed by acid hydrolysis and characterization of the released fragments¹⁻⁴.

Using trifluoroacetolysis it is possible to isolate N- and O-glycosidically linked oligosaccharide chains in glycoproteins⁵⁻⁷. The glycosidic bonds are stable due to the electron withdrawing character exerted by the introduced O-trifluoroacetyl groups in the sugar moiety⁸⁻¹². We now report on a new procedure for the isolation of the oligosaccharides from the carbohydrate-protein linkage region of chondroitin sulphate. The method is based on cleavage of the carbohydrate-protein linkage and degradation of the polysaccharide chains by trifluoroacetolysis¹³.

EXPERIMENTAL

Materials. - Chondroitin 4-sulphate was prepared from bovine nasal septa as described². β -D-Galp-(1-4)-D-Xylp and β -D-Galp-(1-3)- β -D-Galp-(1-4)-D-Xylp were gifts of professor Bengt Lindberg and had been synthesised as described¹⁴.

General methods. - G.l.c. was performed with a Perkin-Elmer 3920 gas chromatograph equipped with a flame ionisation detector. Separations were performed on (a) a SE-30 W.C.O.T. glass capillary column (25 m x 0.25 mm) (Scientific Glass Engineering Inc., USA) at 180-330° (for permethylated oligosaccharide alditols), and (b) a glass column (2 m x 0.5 cm) packed with 3% of ECNSS-M on Chromosorb Q (Applied Science Laboratories Inc., USA) at 200° (for alditol acetates). G.l.c.-m.s. was performed on a Varian MAT 311A instrument fitted with the appropriate column. The spectra were recorded at 70 eV, with an ionising current of 3 mA and an ion-source temperature of 120°. The spectra were processed by an on-line computer system (Spectrosystem 100, Varian MAT).

Analytical methods. - Total hexose was determined with a colorimetric method¹⁵. Sugar analysis was performed by g.l.c.¹⁶ and m.s.¹⁷ after hydrolysis with 90% aqueous formic acid at 100° for 5 h followed by hydrolysis with 0.25M aqueous sulphuric acid at 100° for 18 h. Methylation analysis was performed as previously described¹⁸.

Trifluoroacetylisis. - Chondroitin 4-sulphate (50 g) was heated in a mixture of trifluoroacetic acid (TFA) and

trifluoroacetic anhydride (TFAA) (1:50, 1500 mL) at 100° for 48 h in a sealed vessel, yielding a black solution, (caution, corrosive mixture under pressure). The reaction mixture was cooled to room temperature, concentrated to dryness, dissolved in methanol (500 mL) and again concentrated to dryness. The residue was then treated with 50% aqueous acetic acid (500 mL) for 4 h at room temperature, and concentrated to dryness. One percent (wt/v) of the residue was reduced with sodium borodeuteride (100 mg) and permethylated¹⁹. The permethylated product was analysed by g.l.c.-m.s. The rest of the material was dissolved in water (200 mL) and passed through columns (60 cm x 5 cm) of Dowex 50 (H[⊕]) resin and Dowex 2 (OAc[⊖]) resin (Dow Chemical Company, USA). The desalted solution was concentrated to dryness and fractionated by preparative paper chromatography (ethylacetate/water/acetic acid, 3:1:1). The fractionation was monitored by g.l.c.-m.s. analysis of each fraction after reduction with NaBD₄ and permethylation. The fraction containing β -D-Gal-(1-3)- β -D-Galp-(1-4)-D-Xylp was eluted from the paper and passed through a Sephadex G-25 (Pharmacia Fine Chemicals, Sweden) column (20 cm x 2 cm) eluted with water. The fractions containing the trisaccharide were pooled and lyophilized. The yield of the trisaccharide was 50 mg.

RESULTS

Two separate chondroitin 4-sulphate preparations (6.85 g and 50 g) were used in the degradation by trifluoro-

acetolysis. Analysis of D-xylose present by g.l.c. as alditol acetates showed 0.24% (wt/wt) in both preparations. The recovery β -D-Galp-(1-3)- β -D-Galp-(1-4)-D-Xylp after trifluoroacetolysis of the chondroitin 4-sulphate preparations were 19% (wt/wt) and 14% (wt/wt), respectively. The values are calculated from the analysed yield of D-xylose. Total recovery of the linkage region oligosaccharides were 46% (wt/wt) and 40% (wt/wt), respectively.

G.l.c.-m.s. analyses of the reduced and permethylated products of trifluoroacetolysed chondroitin 4-sulphate gave the ion chromatogram shown in Fig. 1. The ion tracing for four of the major components (peak a-d) indicated that they were derived from the carbohydrate-protein linkage region of the polysaccharide and were further characterized.

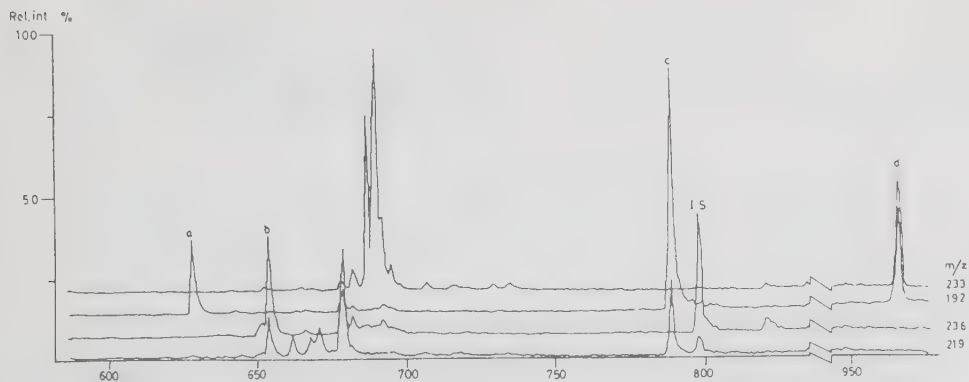


Fig. 1
Multiple ion tracing of g.l.c.-m.s. analysis of permethylated oligosaccharide alditols (peak a-d) released by trifluoroacetolysis of chondroitin 4-sulphate. I.S., internal standard (maltotriose).

The material in peak a gave a mass spectrum (Fig. 2) characteristic of a hexp-(1-4)-pentitol-1-d²⁰. The inten-

sity ratio of the A_1 -, A_2 - and A_3 -fragments can be used to differentiate the gluco-configuration from the manno- and galacto-configurations²¹. The mass spectrum (Fig. 2) indicated that the terminal non-reducing sugar was galactose. Since the only hexose and pentose constituents of chondroitin 4-sulphate are D-galactose and D-xylose, respectively, it was concluded that peak a contained permethylated D-Galp-(1-4)-D-xylitol-1-d. The proposed structure was further supported by the observation that the g.l.c. retention time of the compound was the same as that of an authentic sample of β -D-Galp-(1-4)-D-Xylp which had been reduced and permethylated. The g.l.c. analysis further suggested that the anomeric configuration of the glycosidic linkage was β , since separation of the α and β anomers would likely have occurred.

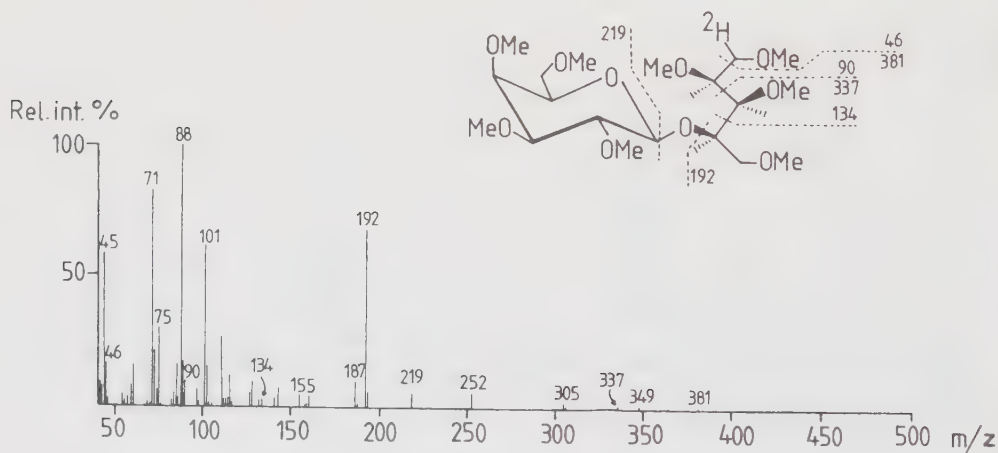


Fig. 2
Mass spectrum and some important primary fragments
of the component in peak a (Fig. 1).

The product in peak b showed a mass spectrum consistent with a permethylated hexp-(1-3)-hexitol-1-d (Fig. 3). Since the only hexose present in chondroitin 4-sulphate is

D-galactose and two β -linked residues of this sugar are present in each polysaccharide molecule, the isolated disaccharide was assumed to be permethylated β -D-Galp-(1-3)-D-galactitol-1-d²¹.

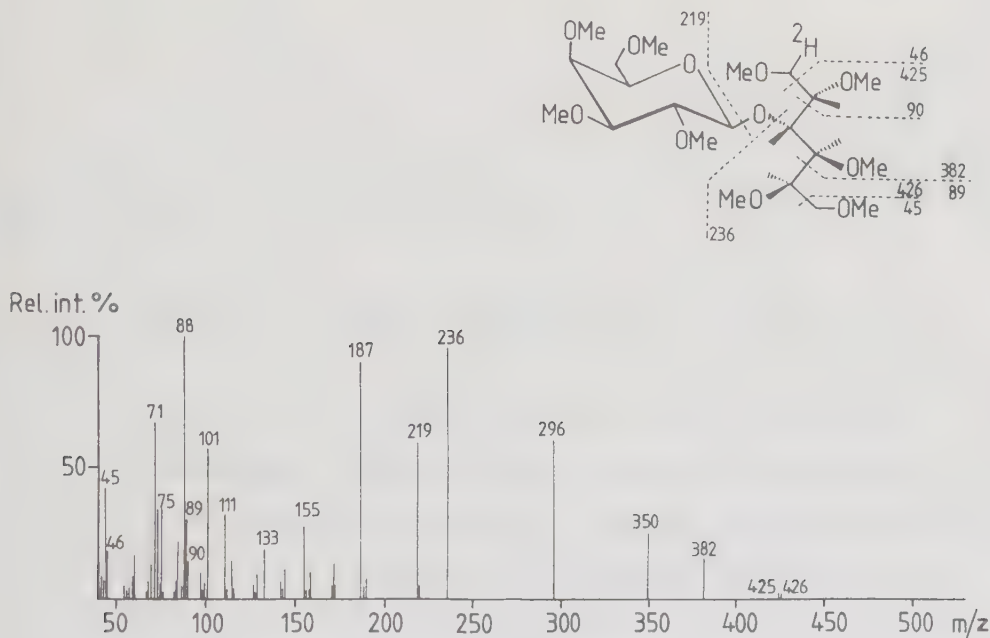


Fig. 3

Mass spectrum and some important primary fragments of the component in peak b (Fig. 1).

The mass spectrum of the major peak, (peak c, Fig. 1) was typical of a permethylated hexp-(1-3)-hexp-(1-4)-pentitol-1-d (Fig. 4). This spectrum was identical to that observed for a reduced and permethylated sample of authentic β -D-Galp-(1-3)- β -D-Galp-(1-4)-D-Xylp. Identity of the oligosaccharide in peak c with the derivative of the authentic trisaccharide was further indicated by the finding that their retention times on g.l.c. were the same.

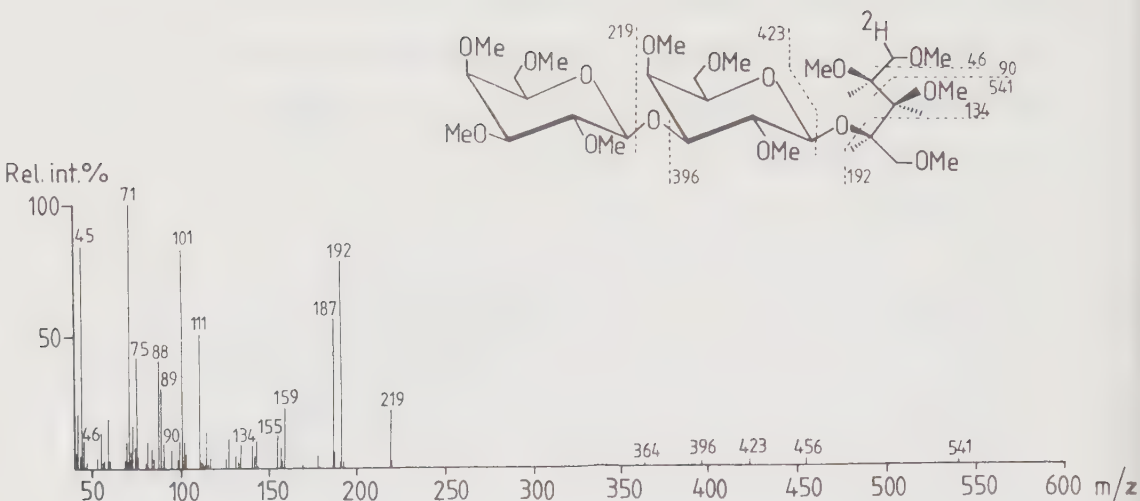


Fig. 4
Mass spectrum and some important primary fragments
of the component in peak c (Fig. 1).

The largest compound (peak d, Fig. 5) showed a mass spectrum characteristic of a permethylated hexpA-(1-3)-
-hexp-(1-3)-hexp-(1-4)-pentitol-1-d, indicating that it

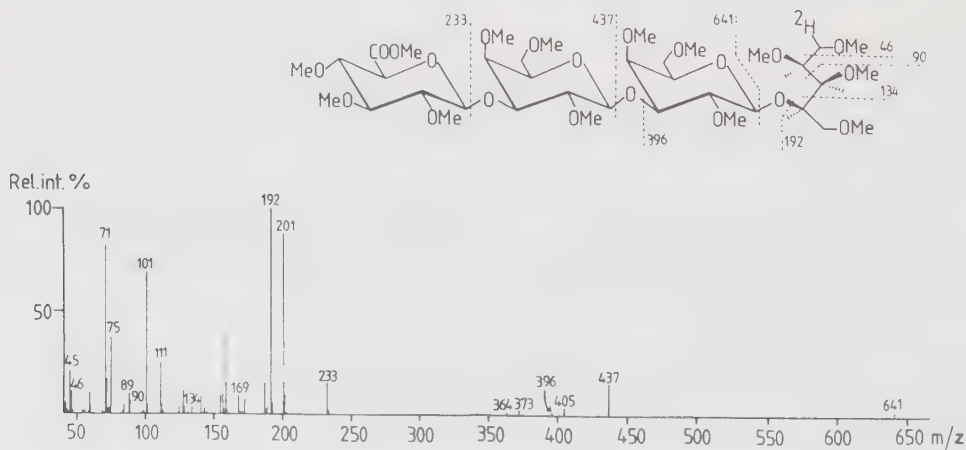


Fig. 5
Mass spectrum and some important primary fragments
of the component in peak d (Fig. 1).

represented the entire linkage region tetrasaccharide and was the permethylated derivative of β -D-GlcpA-(1-3)- β -D-Galp-(1-3)- β -D-Galp-(1-4)-D-xylitol-1-d. The intensity ratio of the A-fragments of hexuronic acids show the same relationship as for neutral hexoses and indicated a terminal non-reducing D-GlcpA²¹.

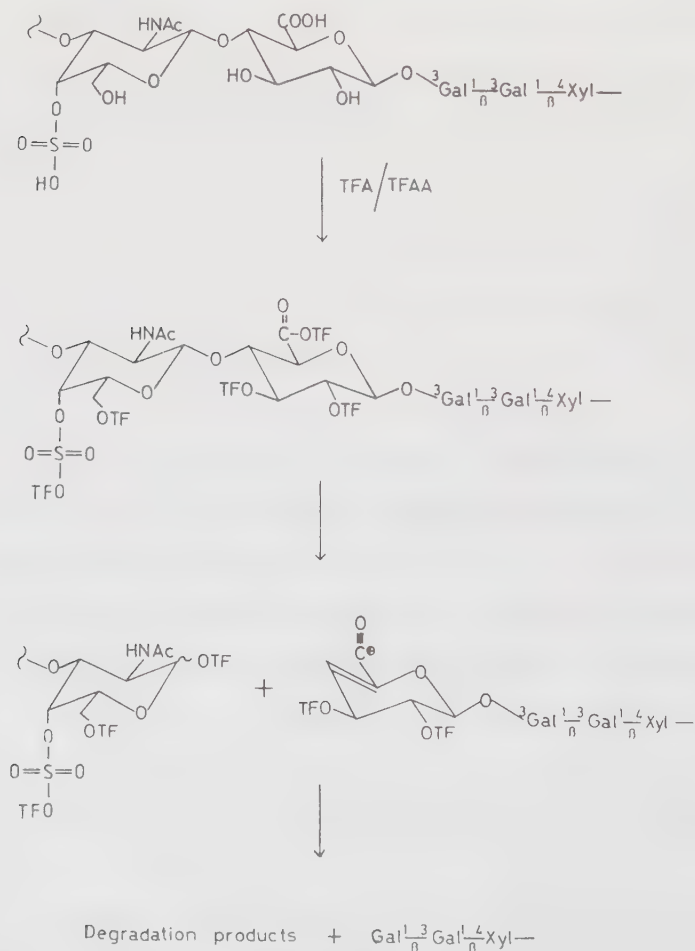
The response of m/z 233 at scan 680 in the ion chromatogram (Fig. 1) correspond to degradation products of the polysaccharide chains under trifluoroacetolysis.

Further characterization of the major product of the trifluoroacetolysis was carried out as follows. The bulk (99%) of the reaction mixture after removal of the trifluoroacetyl groups was desalted and subjected to preparative paper chromatography, yielding 50 mg of trisaccharide (β -D-Galp-(1-3)- β -D-Galp-(1-4)-D-Xylp) from 50 g of chondroitin 4-sulphate. Monosaccharide composition was analysed by g.l.c. as alditol acetates and showed D-galactose and D-xylose in a molar ratio of 2:1. The fraction containing the trisaccharide was pure as determined by g.l.c.-m.s. after reduction with NaBD_4 and permethylation. Methylation analysis of the reduced trisaccharide gave 2,3,4,6-tetra-O-methyl-D-Gal, 2,4,6-tri-O-methyl-D-Gal and 1,2,3,5-tetra-O-methyl-D-Xylitol-1-d in equimolar proportions.

DISCUSSION

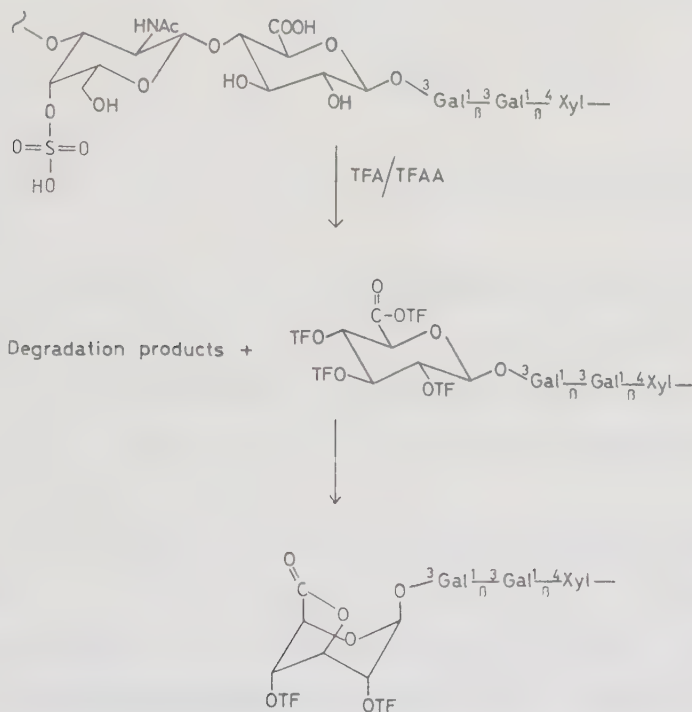
Trifluoroacetolysis of chondroitin 4-sulphate results in release of the oligosaccharide chains from the protein

core by acid catalyzed elimination⁶ after degradation of the protein by transamidation⁵. The disaccharide repeating unit, $-3)-(SO_4^{\ominus}-4)-\beta\text{-}\underline{\text{D}}\text{-GalNAc}\text{-}(1-4)-\beta\text{-}\underline{\text{D}}\text{-Glc}\text{pA}-$ (1-, is degraded by solvolysis of both the glycosidic linkages. The glycosidic linkage of the 4-O-sulphated D-GalNAc residue is cleaved due to lack of protection by O-trifluoroacetyl groups, and the glycosidic linkage of the D-Glc pA residue is cleaved after acid catalyzed elimination of the substituent in position 4 (Scheme 1).



Scheme 1

However, if the 4-O-sulphated D-GalNAcp residue is solvolysed, a nonreducing terminal D-Glc_pA residue is generated, which to a large extent is stable towards trifluoroacetylation because of lactone formation¹³ (Scheme 2).



Scheme 2

The trisaccharide β -D-Galp-(1-3)- β -D-Galp-(1-4)-D-Xylp is essentially stable after being pertrifluoroacetylated due to the electron withdrawing power exerted by the O-trifluoroacetyl groups^{6,8}. The formation of a small amount of β -D-Galp-(1-3)-D-Galp (peak b, Fig. 1) may be ascribed to some degradation by elimination reactions⁶. However, the presence of β -D-Galp-(1-4)-D-Xylp (peak a, Fig. 1) among the reaction products was not expected and cannot readily be

explained by degradation of the trisaccharide. Although there is a remote possibility that some polysaccharide chains contained an incomplete linkage region with only one D-galactose residue, previous quantitative analyses of glycopeptides from the linkage region indicate that such incomplete structures do not exist in normal cartilage³.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council (03X-4956), the Medical Faculty, University of Lund, and the National Institutes of Health (DE 2670).

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SPECIFIC CLEAVAGE OF THE GLYCOSIDIC BOND BETWEEN THE
CARBOHYDRATE AND CERAMIDE PORTIONS IN GLYCOSPHINGOLIPIDS
USING TRIFLUOROACETOLYSIS*

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* The principle and part of this work was presented at
the C.N.R.S. Internat. Symp. on Structure and Function of
Gangliosides. Svensson, S. 1980 (EDS. Svennerholm, L.,
Mandel, P., Dreyfus, M. and Urban, P.-F., Plenum Press, New
York) pp. 71-76.

SUMMARY

A number of glycosphingolipids have been subjected to
trifluoroacetolysis. Heating the glycosphingolipid at 100
°C for 48 h in mixtures of trifluoroacetic acid and tri-
fluoroacetic anhydride varying from 1:1 to 1:100 (v/v) re-

sulted in complete cleavage of the glycosidic bond between the carbohydrate and the unsaturated base of the ceramide. The fatty acid(s) linked to the amino group of sphingosine were also released by transamidation. The carbohydrate portion was stable under these conditions due to stabilization of the glycosidic bonds by the electronegative O-trifluoroacetyl groups. The 2-acetamido-2-deoxy-hexose units in the glycosphingolipids were converted into their N-trifluoroacetyl analogues. The N-trifluoroacetyl functions could be removed and the 2-acetamido-2-deoxy-hexoses could be reconstituted by N-acetylation.

INTRODUCTION

In previous publications we have demonstrated the versatility of the trifluoroacetolysis reaction. By trifluoroacetolysis it is possible to effect transamidation under conditions where most glycosides and reducing sugars are stable, being converted into their pertrifluoroacetylated derivatives¹⁻⁵. By use of the trifluoroacetolysis reaction, procedures have been devised for the isolation of O- and N-glycosidically linked carbohydrate chains in glycoproteins⁶⁻⁹. Trifluoroacetolysis has also been used for releasing oligosaccharides from erythrocyte membranes of different blood group P phenotypes¹⁰. In this communication glycosphingolipid models have been subjected to trifluoroacetolytic degradation.

MATERIALS AND METHODS

Glycosphingolipids - Glucosylceramide, galactosylceramide, lactosylceramide and GM₁-ganglioside were purchased from Supelco Inc. (USA). Globotriaosylceramide was prepared from pig intestinal linings¹¹ and the glycosphingolipids gangliotriaosylceramide, globotetraosylceramide and the Forssman antigen were prepared from guinea pig¹², human¹³ and sheep¹⁴ erythrocyte membranes, respectively. The identity of the glycosphingolipids was established by sugar analysis, methylation analysis and ¹H NMR spectroscopy.

Analytical methods - Sugar analyses were performed using gas-liquid chromatography¹⁵ and mass spectrometry¹⁶ after hydrolysis with 90% aqueous formic acid at 100 °C for 5 h and also after subsequent hydrolysis with 0.25M aqueous sulphuric acid at 100 °C for 18 h. Methylation analyses were performed as previously described¹⁷. Gas-liquid chromatography (GLC) was carried out on a Perkin-Elmer 3920 gas chromatograph equipped with a flame ionisation detector. Separations were performed on (a) a SE-30 W.C.O.T. vitreous silica capillary column (25m x 0.2mm) at 170 °C (for partially methylated alditol acetates) and at 180-330 °C (for permethylated oligosaccharides alditols), (b) a Silar 10 W.C.O.T. glass capillary column (4m x 0.25mm) at 190-230 °C (for alditol acetates). Gas-liquid chromatography-mass spectrometry (GLC-MS) was performed on a Finnigan 4021 instrument fitted with the appropriate column. The

spectra were recorded at 70 eV, with an ionisation current of 0.3 mA and an ion source temperature of 280 °C. The spectra were processed on an on-line computer system (Nova 3, Data General).

Trifluoroacetylolysis of glycosphingolipids - The glycosphingolipids were treated with a mixture of trifluoroacetic acid (TFA) and trifluoroacetic anhydride (TFAA) at 100 °C in a sealed tube (caution: corrosive mixture under pressure). The reaction time was varied from 2 to 48 h and the proportions of TFA/TFAA (2 ml/mg glycosphingolipid) was varied from 1:1 to 1:100 (v/v). After cooling, the reaction mixture was concentrated to dryness, dissolved in methanol (5 ml) and again concentrated to dryness. The product was dissolved in glacial acetic acid (5 ml) and distilled water (5 ml) was added. After 30 min at 100 °C the solution was cooled and evaporated to dryness. The released oligosaccharides were freed from contaminating material by extraction with diethyl ether (10 ml) and distilled water (5 ml). The water phase was collected and the diethyl ether phase was shaken two more times with 5 ml water. The combined water phases were washed three times with 5 ml diethyl ether and evaporated to dryness.

Oligosaccharides from glycosphingolipids containing 2-acetamido-2-deoxy-hexoses were reconstituted by reduction (NaBD_4) of the material and subsequent N-acetylation. The N-acetylation was performed in two steps, first acetylation by acetic anhydride/pyridine (1:1, (v/v), 5 ml, 100 °C, 30 min) and then de-O-acetylation with 2M ammonia in 75 % aqueous methanol (10 ml) at room temperature for 20 h.

RESULTS AND DISCUSSION

Trifluoroacetolysis of the simple glycosphingolipids glucosylceramide, galactosylceramide, LcOse_2Cer and GbOse_3Cer proceeded well in mixtures of TFA/TFAA varying from 1:1 to 1:100 (v/v). After work up the undegraded oligosaccharides were recovered in high yield and analysed by GLC-MS as alditol acetates for the monosaccharides and as permethylated alditols for the oligosaccharides (Table 1). The reaction time needed for accomplishing complete cleavage of the glycosidic bond between the sugar moiety and the ceramide portion was 21 and 27 h respectively for the trifluoroacetolysis conditions 1:1 and 1:50 (v/v) (Fig. 1).

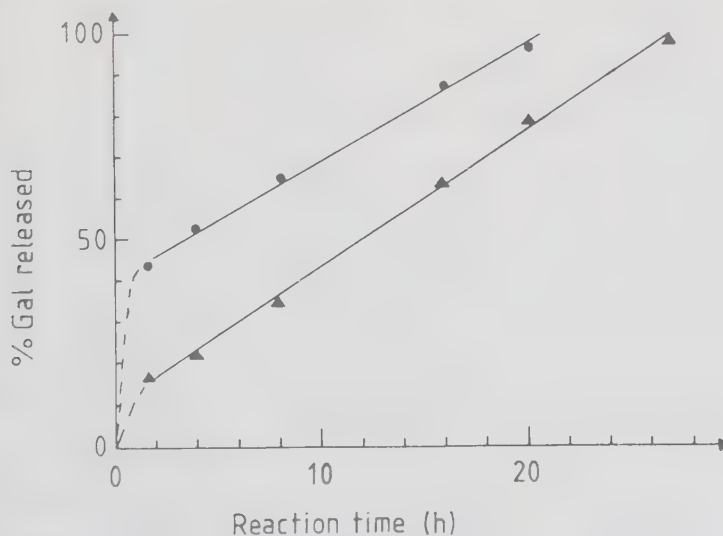


Fig. 1

Treatment of galactosylceramide with TFA/TFAA 1:1 (—●—) and TFA/TFAA 1:50 (—▲—) (v/v).

The cleavage mechanism is possibly initiated by elimina-

Table 1. Treatment of glycosphingolipids with TFA/TFAA at 100° C.

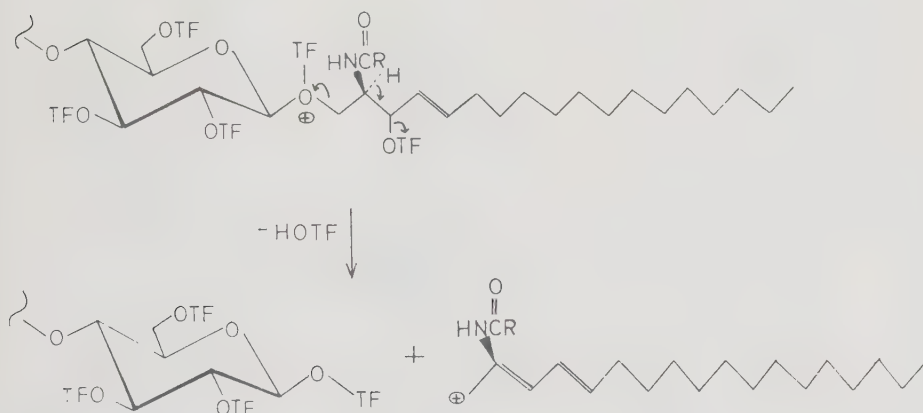
Compound	Product ^b	Recovery% ^a			
		TFA/TFAA 24h		TFA/TFAA 48h	
		1:1	1:50	1:1	1:50
					1:100
Glc-Cer	D-Glc-ol-1-1-d ^c	97	89	98	98
Gal-Cer	D-Gal-ol-1-1-d	100	91	97	99
LcOse ₂ Cer	β-D-Gal-(1-4)-D-Glc-ol-1-1-d	97	87	97	98
GbOse ₃ Cer	α-D-Gal-(1-4)-β-D-Gal-(1-4)-D-Glc-ol-1-1-d	98	88	96	97

^a Determined by GLC-MS, as alditol acetates for the monosaccharides and as permethylated oligosaccharide alditols for the others.

^b Product obtained after trifluoroacetolysis, de-O-trifluoroacetylation and reduction (NaBD₄)

^c D-Glc-ol-1-1-d, D-glucitol-1-1-d

tion of an O-trifluoroacetyl group from the sphingosine unit yielding an allyl cation (Scheme 1). This mechanism requires that the sphingosine moiety is unsaturated as previously has been suggested¹⁸.



Scheme 1

Trifluoroacetolysis of $GgOse_3Cer$ and $GbOse_4Cer$ was performed in two mixtures of TFA/TFAA, 1:1 and 1:50 (v/v), and at both conditions the oligosaccharide portions were obtained in good yields as their pertrifluoroacetylated derivatives. After reconstitution (see Materials and Methods) the oligosaccharides were analysed by GLC-MS as their permethylated oligosaccharide alditols (Table 2, Fig 2 and 3).

The Forssman antigen was subjected to trifluoroacetolysis in different mixtures of TFA/TFAA varying from 1:1 to 1:100 (v/v). In TFA/TFAA mixtures varying from 1:1 to 1:15 (v/v) some cleavage of the bond of the innermost D-GalNAc residue was observed, yielding on analysis the disaccharide α -D-GalNAc-(1-3)-D-GalNAc and the trisaccharide α -D-Galp-(1-4)- β -D-Galp-(1-4)-D-Glc as well as the undegraded pentasaccha-

Table 2. Treatment of glycosphingolipids with TFA/TFAA at 100° C for 48 h.

Compound	Product ^b	Recovery% ^a	
		TFA/TFAA 1:1	TFA/TFAA 1:50
GgOse ₃ Cer	β-D-GalNac-(1-4)-β-D-Gal-(1-4)-D-Glc-ol-1-d ^c	98	100
GbOse ₄ Cer	β-D-GalNac-(1-3)-α-D-Gal-(1-4)-β-D-Gal-(1-4)-D-Glc-ol-1-d	87	89

^a Determined by GLC-MS, as permethylated oligosaccharide alditols.

^b Product obtained after trifluoroacetytolysis, de-0- and de-N-trifluoroacetylation with NaBD₄ and N-acetylation.

^c D-Glc-ol-1-d, D-glucitol-1-d.

Table 3. Treatment of Forssman antigen with TFA/TFAA at 100° C for 48 h.

Product ^b	Recovery% ^a	
	TFA/TFAA 1:1	TFA/TFAA 1:50
α-D-Gal-Nac-(1-3)-β-D-GalNac-(1-3)-α-D-Gal-(1-4)-β-D-Gal-(1-4)-D-Glc-ol-1-d ^c	28	67
α-D-Gal-Nac-(1-3)-β-D-GalNac-(1-3)-α-D-Gal-(1-4)-β-D-Gal-(1-4)-D-Glc-ol-1-d ^c	26	83

^a Determined by paper chromatography and also by GLC-MS, where the relationship terminal galactose and 3-linked galactose was calculated.

^b Product obtained after trifluoroacetytolysis, de-0- and de-N-trifluoroacetylation with NaBD₄ and N-acetylation.

^c D-Glc-ol-1-d, D-glucitol-1-d.

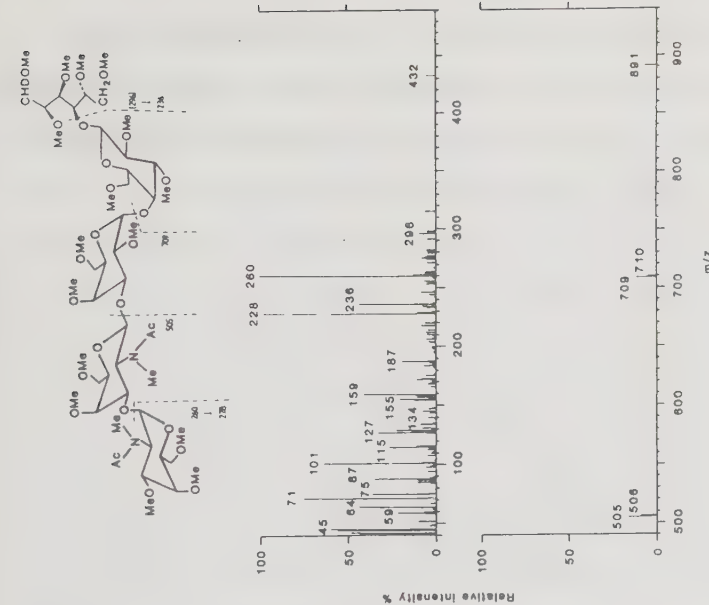


Fig. 4

Mass spectrum and some important primary fragments of α -D-GalNAc-(1-3)- β -D-GalNAc-(1-3)- α -D-Gal-(1-4)- β -D-Gal-(1-4)-D-Glc-ol-1-d as permethylated oligosaccharide alditol released from Forssman antigen by trifluoroacetytolysis.

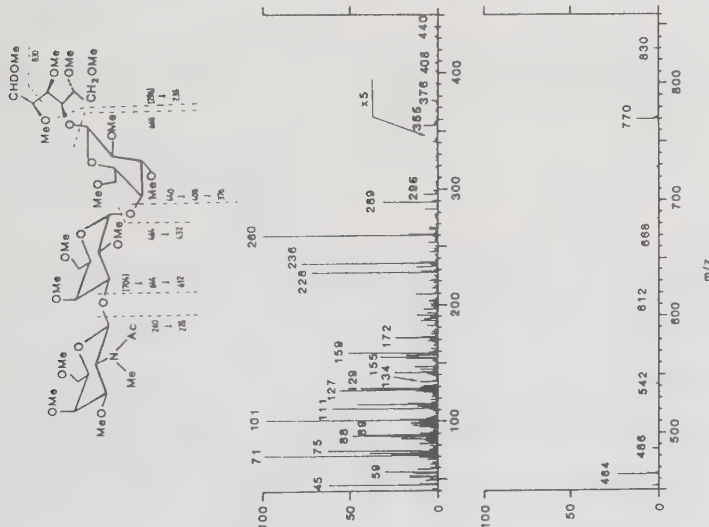


Fig. 3

Mass spectrum and some important primary fragments of β -D-GalNAc-(1-3)- α -D-Gal-(1-4)- β -D-Gal-(1-4)-D-Glc-ol-1-d as permethylated oligosaccharide alditol released from GbOse₄Cer by trifluoroacetytolysis.

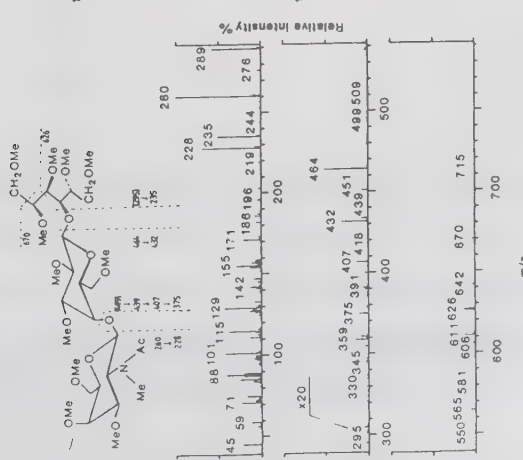


Fig. 2

Mass spectrum and some important primary fragments of β -D-GalNAc-(1-3)- α -D-Gal-(1-4)-D-Glc-ol as permethylated oligosaccharide alditol released from GbOse₃Cer by trifluoroacetytolysis.

ride α -D-GalNAcp-(1-3)- β -D-GalNAcp-(1-4)- α -D-Galp-(1-4)- β -D-Galp-(1-4)-D-Glc (Table 3). Using TFA/TFAA mixtures varying from 1:50 to 1:100 (v/v) the expected pentasaccharide was obtained in good yield as shown (Table 3) by paper chromatography after removal of trifluoroacetyl groups and subsequent N-acetylation. The pentasaccharide was isolated and subjected to sugar- and methylation analysis (Table 4) and the structure was verified by direct probe MS of the reduced and permethylated compound (Fig. 4). The anomeric nature of the sugar residues as well as the number of N-acetyl groups were established by ^1H NMR spectroscopy.

Table 4. Sugar and methylation analysis of the oligosaccharide released from Forssman antigen by trifluoroacetolysis

Sugars	Relative molar proportions ^a
<u>D</u> -Gal _b	1.85
<u>D</u> -Glc _b	1.0
<u>D</u> -GalNAc	1.65

^a Determined by GLC-MS after hydrolysis, reduction and acetylation

^b D-Glc is set to 1.0

Sugars	Relative molar proportions ^c
1,2,3,5,6- <u>O</u> -Me- <u>D</u> -Glc	0.97
2,3,6- <u>O</u> -Me- <u>D</u> -Gal ^d	1.0
2,4,6,- <u>O</u> -Me- <u>D</u> -Gal	0.82
3,4,6- <u>O</u> -Me- <u>D</u> -GalN(Me)Ac	] 0.85 ^e
3,4,6- <u>O</u> -Me- <u>D</u> -GalNAc	
4,6,- <u>O</u> -Me- <u>D</u> -GalN(Me)Ac	] 0.80 ^e
4,6- <u>O</u> -Me- <u>D</u> -GalNAc	

^c Determined by GLC-MS after permethylation, hydrolysis, reduction and acetylation

^d 2,3,6-O-Me-D-Gal is set to 1.0

^e The relative molar proportions were calculated relative the amount of D-Gal from the sugar analysis, since the re-sponsfactors for the hexosamine derivatives are not known

These results show that the glycosidic bond between a sugar residue and the ceramide portion with an unsaturated sphingosine in glycolipids can be cleaved under conditions where most glycosidic bonds are stable and amides are transamidated into N-trifluoroacetyl functions. By de-O- and de-N-trifluoroacetylation followed by re-N-acetylation the carbohydrate portion of neutral glycosphingolipids can be recovered intact. The methodology is presently being explored for use in the development of a diagnostic procedure for the sphingolipidoses, as well as for preparative techniques for isolation of the oligosaccharide portions in glycosphingolipids.

ACKNOWLEDGMENTS

The authors are indebted to Mrs Lisbeth Palm-Svensson and Mrs Christin Lundsten for excellent technical assistance and to the Swedish Medical Research Council (03X-4956) and the Magnus Bergvall's Foundation for financial support.

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STRUCTURAL STUDIES OF THE O-GLYCOSIDICALLY LINKED
CARBOHYDRATE CHAINS OF GLUCOAMYLASE G1 FROM ASPERGILLUS NIGER

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SUMMARY

Glucoamylase G1 from Aspergillus niger contains an unusual type of carbohydrate-protein linkage, involving mannose O-glycosidically linked to serine and threonine. The majority of the neutral carbohydrates of glucoamylase G1 are located in a region of about 70 amino acid residues which carries about 35 oligosaccharide units [Carlsberg Res. Commun. 48, 517-527, (1983)]. Structural analysis was performed on the O-linked carbohydrates of a tryptic fragment from glucoamylase G1 comprising the segment characterized by a high degree of glycosylation. The carbohydrate structures released by trifluoroacetolysis were elucidated using sugar analysis,

methylation analysis, mass spectrometry, chromium trioxide oxidation and digestion with α -mannosidase. The following structures could be identified:

Man 1 - 0 - Ser/Thr

Man α 1 - 2 Man 1 - 0 - Ser/Thr

Man α 1 - 6 Man 1 - 0 - Ser/Thr

Man α 1 $\begin{array}{l} \diagdown \\ 2 \\ \diagup \end{array}$ Man 1 - 0 - Ser/Thr
 Man α 1 $\begin{array}{l} \diagdown \\ 6 \\ \diagup \end{array}$

At least one more hexose containing disaccharide, 1-6 linked was also observed, but the identity of the monosaccharide moieties have not fully been established.

INTRODUCTION

Glucoamylase G1 (EC 3.2.1.3) is an exohydrolase which catalyzes the release of glucose from the non-reducing end of starch and related oligo- and polysaccharides [1-3]. The enzyme is of great industrial importance and is produced in large scale by various fungi [4,5]. The glucoamylase G1 from Aspergillus niger is an unusual glycoprotein characterized by a large number of short 0-glycosidically linked carbohydrate chains [6,7]. The amino acid sequence of G1 was recently determined [8]. The enzyme comprises 616 amino acid residues and 19% (wt/wt) of neutral carbohydrates of which the majority are located in a segment of 70 amino acids [8,9]. Only a few amino acids outside this glycosylated region are assigned to carry carbohydrates, among these are two

N-glycosylated asparagine residues [8,9]. Structural studies of the O-linked oligosaccharides of glucoamylase G1 has previously been reported by Pazur et al. [7]. The present study reports on the structure of the O-glycosidically linked carbohydrate units of the highly glycosylated region of glucoamylase G1. The structures are different from those found by Pazur et al. [7].

EXPERIMENTAL PROCEDURES

Materials - Glucoamylase G1 from Aspergillus niger was prepared as previously described by Svensson et al. [10] from AMG 200L produced by Novo Industri, Bagsvaerd, Denmark. The tryptic fragment, Tca2-1, was isolated according to published procedure [9] from 2-pyridylethylated and citraconylated glucoamylase G1. Trifluoroacetic acid (TFA) and trifluoroacetic anhydride (TFAA) were from Ega-Chemie, F.R.G. α -Mannosidase from jack bean was kindly provided by Dr. Alan Chester (University of Lund). All other reagents were of reagent grade or higher quality.

Analytical Methods - Monosaccharides were analysed as alditol acetates by gas-liquid chromatography (GLC) [11] and mass spectrometry (MS) [12] after hydrolysis with 90% aqueous formic acid at 100⁰ C for 5 h followed by hydrolysis with 0.25M sulphuric acid at 100⁰ C for 18 h. Methylation analyses were performed as previously described [13]. GLC was carried out on a Perkin-Elmer 3920 gas chromatograph equipped with a flame ionisation detector. Separations were performed

on (a) a SE-30 W.C.O.T. vitreous silica capillary column (25 m x 0.2 mm) at 180⁰ C for partially methylated alditol acetates and at 180-330⁰ C for permethylated oligosaccharide alditols, (b) a Silar 10 glass capillary column (4 m x 0.25 mm) at 170-210⁰ C for alditol acetates. GLC-MS was performed on a Finnigan 4021 instrument fitted with the appropriate column. The spectra were recorded at 70 eV, with an ionisation current of 0.3 mA and an ion-source temperature of 280⁰ C. The spectra were processed on an on-line computer system (Nova 3, Data General).

Trifluoroacetolysis [14] - To 7 mg glycopeptide Tca2-1 was added 25 ml of TFA/TFAA 1:50 (v/v). The solution was heated to 100⁰ C for 48 h in a sealed thick-walled glasstube. (Caution - highly corrosive mixture under pressure). The mixture was cooled to room temperature and concentrated to dryness by rotary evaporation. O-Trifluoroacetyl groups were removed by evaporation from methanol twice (2x5 ml).

α -Mannosidase treatment - Oligosaccharides (0.9.mg) released by trifluoroacetolysis, from the tryptic fragment Tca2-1 were dissolved in 1 ml of 0.05M sodium acetate buffer, pH 5.0 containing 0.1M NaCl and 0.1mM zink acetate. To the solution was added 0.5 ml α -mannosidase (12 U/ml). The digest was left at room temperature over night with stirring. The enzyme was denaturated by adding 1 ml H₂O and heating to 100⁰ C for 10 min. After centrifugation the supernatant was desalted on columns of Dowex 50 resin (H⁺) and AG 3 (OAc⁻) resin.

Chromium trioxide oxidation [15] - Oligosaccharides (0.2 mg) released by trifluoroacetolysis, from the tryptic

fragment Tca2-1 were reduced and acetylated using acetic anhydride/pyridine (2:1 (v/v), 3 ml, 30 min, 100° C). The acetylated oligosaccharide mixture, together with xylitol pentaacetate (internal standard), was dissolved in 0.1 ml of glacial acetic acid and 10 mg of powdered chromium trioxide was added. The suspension was sonicated at 50° C for 1 h, and two ml of water was added and the material was recovered by extraction into chloroform. The chloroform phase was concentrated to dryness and subjected to sugar analysis.

RESULTS

The carbohydrate content of the tryptic glycopeptide, Tca2-1, obtained from glucoamylase G1, was 50% (wt/wt) as determined by GLC as alditol acetates. Table 1 shows the monosaccharide composition in number of residues per mole glycopeptide. Man and Glc were the dominating monosaccharides. Only trace amount of Gal and no aminosugars were detected in Tca2-1. The absence of aminosugars indicate an unusual carbohydrate-protein linkage not commonly found in glycoproteins.

It has previously been shown by Pazur et al. [7] that some of the carbohydrates in glucoamylase can be released by mild alkaline borohydride treatment (Carlsson degradation, [16]), which indicated that they are O-glycosidically linked to serine or threonine. We have previously shown that O-glycosidically as well as N-glycosidically linked carbohydrates can be isolated after trifluoroacetolysis [14,17]. Treatment

of the Tca2-1 glycopeptide with trifluoroacetolysis liberated the carbohydrates intact from the peptide moiety. Sugar analysis after such a treatment gave about the same sugar composition as for the intact glycopeptide (Table 1). Furthermore free Man was formed which indicates that Man is linked as monosaccharide residues to the peptide.

Table 1. Sugar analyses of intact glycopeptide Tca2-1 and its trifluoroacetolysis products.

Sugars	moles/mole glycopeptide		
	Intact	TFA/TFAA	
	Tca2-1	A ^a	B
Man	41	38	5
Gal	<1	0	0
Glc	8	7	<1

^a A total monosaccharides, B free monosaccharides

The glucoamylase G1 glycopeptide was subjected to methylation analysis (Table 2). Non-reducing terminal Man, 2-O-substituted Man, 6-O-substituted Man and 2,6-di-O-substituted Man were observed. Methylation analysis after trifluoroacetolysis and reduction confirmed by the hexa-O-methylated derivative of Man-ol that single mannosyl residues are common in glucoamylase. By examining the reduced terminals formed after this treatment it could be concluded that oligosaccharides containing 2-O-substituted Man, 6-O-substituted

Table 2. Methylation analyses of the glycopeptide Tca2-1 and its trifluoroacetolysis products.

	mol(%) of total carbohydrates	
	Tca2-1	TFA/TFAA
1,2,3,4,5,6- <u>O</u> -Me-Man	0	+ ^a
1,3,4,5,6- <u>O</u> -Me-Man	0	+
1,2,3,4,5- <u>O</u> -Me-Man	0	+
1,3,4,5- <u>O</u> -Me-Man	0	+
2,3,4,6- <u>O</u> -Me-Man	} 64	} 78
2,3,4,6- <u>O</u> -Me-Glc		
3,4,6- <u>O</u> -Me-Man	11	0
2,3,4- <u>O</u> -Me-Man	} 6	} 0
2,3,4- <u>O</u> -Me-Glc		
3,4- <u>O</u> -Me-Man	19	3

^a Methyl ether derivative was detected but not quantitated due to its volatility.

Man and 2,6-di-O-substituted Man as their reducing terminals must be present.

The oligosaccharides released by trifluoroacetolysis and subsequent reduction were analysed by GLC-MS as permethylated alditols. Only mono-, di- and trisaccharide alditols were observed. The ion chromatogram of the di- and trisaccharide region is shown in Fig. 1. This analysis is in contrast to the findings of Pazur et al. [7] who found several tetra-saccharides as well. Two disaccharides alditols were identified, by their mass spectra and by comparison with

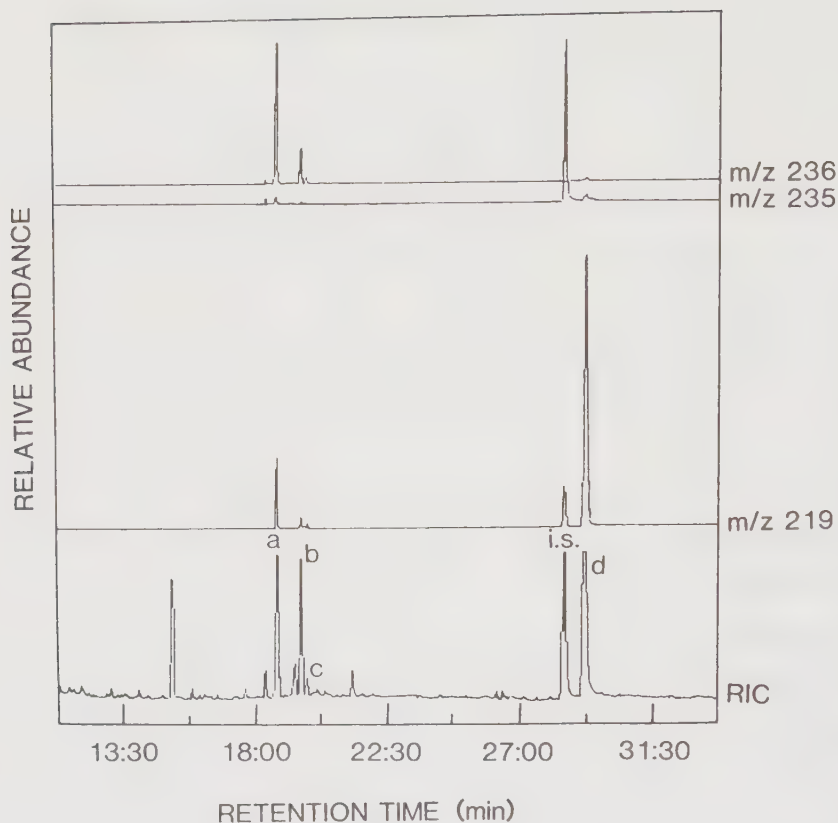


Fig. 1

Ion trace chromatogram of permethylated oligosaccharide alditols. The oligosaccharides were liberated by trifluoroacetolysis from the glycopeptide Tca2-1.

authentic standards, as Man α 1-2 Man-ol and Man α 1-6 Man-ol (peak a and c, Fig. 1). No linear but a branched trisaccharide alditol could also be identified (peak d, Fig. 1). The mass spectrum of the trisaccharide alditol is shown in Fig. 2. A branched alditol can easily be recognized by the absence of m/z 236, which is a strong ion for a mono-O-substituted hexitol-1-d. The presence of ions m/z 219, m/z 440 and the absence of ions for a non-reducing terminal disaccharide

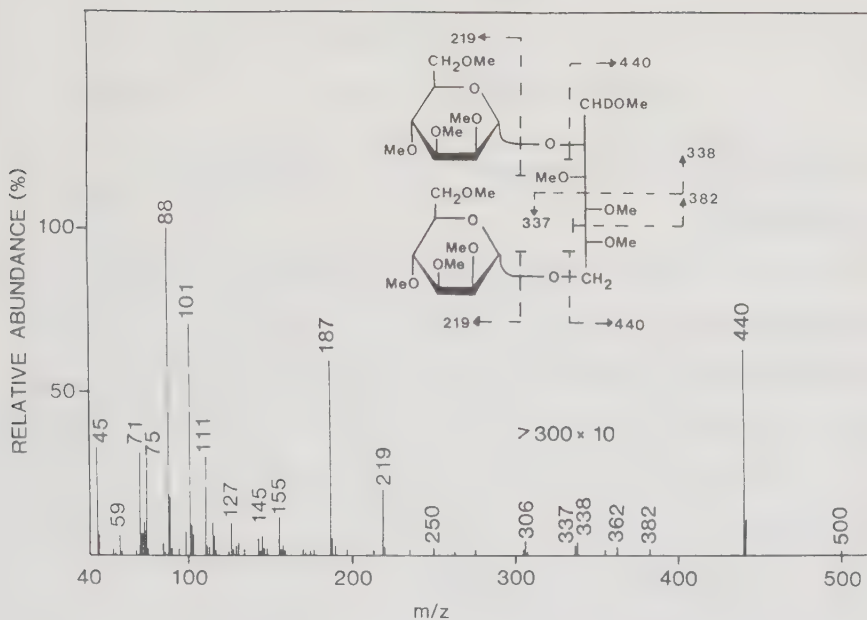
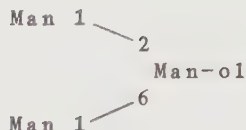


Fig. 2
Mass spectrum of peak d in Fig. 1

sequence show that the alditol is substituted by two hexosyl residues. The positions of substitution of the alditol are determined by ions formed by cleavage within the alditol. The ions m/z 337, m/z 338, and m/z 306 (elimination of methanol from m/z 338) indicate that the alditol is substituted in the 2- and 6-positions. The positions of substitution of the alditol was further confirmed by the methylation analysis of the glycopeptide where a 2,6-di-O substituted Man (3,4-O-Me-Man, Table 2) could be identified. This Man derivative appeared in the methylation analysis after trifluoro-acetolysis and reduction as a 2,6-di-O-substituted Man-ol (1,3,4,5-O-Me-Man, Table 2). The structure of the branched trisaccharide alditol must therefore be,



This branching pattern is unusual for a glycoprotein oligosaccharide.

At least one more disaccharide was observed by GLC-MS after trifluoroacetolysis (peak b, Fig. 1). The mass spectrum of the permethylated derivative showed Hex 1-6 Hex-ol. Glucose is the most likely non-reducing terminal in this disaccharide when its retention time on GLC was the same as for Glc α 1-6 Glc-ol and Glc α 1-6 Man-ol which do not separate on a SE-30 vitreous silica capillary column.

The anomeric configuration was determined by digestion with α -mannosidase and chromium trioxide oxidation. Digestion with α -mannosidase of the oligosaccharide alditols liberated by trifluoroacetolysis of the Tca2-1 glycopeptide released about 80% of the Man. The remaining Man not released by the enzyme could be β -linked or α -linked since a precipitate was formed during the digestion which may prevent the action of α -mannosidase. Carbohydrates liberated by trifluoroacetolysis were reduced, acetylated, and subjected to chromium trioxide oxidation. Sugar analysis after the oxidation showed an insignificant loss of Man. The conclusion must therefore be that at least 80% of the Man is α -linked and presence of β -linked Man is uncertain.

The carbohydrate structures and the number of chains per molecule obtained from the Tca2-1 glycopeptide are summarized in Table 3.

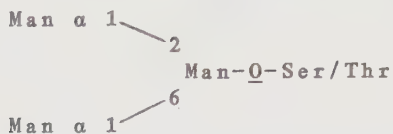
Table 3. Oligosaccharides from glycopeptide Tca2-1 released by trifluoroacetolysis.

Compound	moles/mole glycopeptide
Man	5
Man α 1-2 Man	5
Man α 1-6 Man	<1
Hex 1-6 Hex	2
Man α 1 	11
Man α 1  Man	

DISCUSSION

Glucoamylase G1 from Aspergillus niger contains mannosyl-serine and mannosyl-threonine linkages which are rarely found in glycoproteins [18]. The glycoprotein was previously described to contain a large number oligosaccharides with an average size of two monosaccharide residues [6,7,10]. It has recently been shown that the majority of the neutral carbohydrates of glucoamylase G1 are in a segment containing about 70 amino acids located about 100 amino acid residues from the C-terminus [8]. Mono-, di- and trisaccharides were isolated from a glycopeptide comprising this region of the molecule. Structural analysis revealed that the monosaccharide units were connected by 1-2

and 1-6 linkages. Only one trisaccharide was found:



its branching pattern is unusual for glycoproteins. This type of branched Man has however been reported in N-linked oligosaccharides of tetraantennery type [19] and in O-linked oligosaccharides of yeast cell wall mannan-proteins [20]. Previously Pazur et al. [7] suggested longer oligosaccharides and 1-3 glycosidic linkages. Lineback et al. [6] described unbranched trisaccharides to be the major carbohydrate substituent. The carbohydrate composition from both studies showed Man as dominant and Glc as the only other major monosaccharide in agreement with the present finding. However, discrepancies exist between the structure of the oligosaccharide units observed in all three laboratories. It seems difficult to explain this fact, but presumably different strains and growth conditions may be at least in part responsible.

The glycosidic linkages are probably α in the oligosaccharide chains and this is in agreement with ^{13}C nuclear magnetic resonance spectroscopy studies on glucoamylase from Aspergillus niger by Dill and Allerhand [21].

Two other secreted fungal carbohydrases, mycodextranase from Penicillium melinii [22], and cellobiohydrolase from Trichoderma viride [23], possessed multiple attachment sites for oligosaccharide units of an average size of a disaccha-

ride. In both cases an uneven distribution of the substituents along the polypeptide chain has been suggested. Mycodextranase contained unbranched trisaccharides as the principal carbohydrate units and some mono and disaccharides. The cellobiohydrolase contained from mono up to pentasaccharides, the dominating substituent being unbranched 1-6 linked trisaccharides. The role of the carbohydrate moieties in the three enzymes is still unknown.

The occurrence of a carbohydrate-peptide bond involving mannose and a hydroxyamino acid is rare, but has been reported to exist in collagen from certain earth worms [24]. It has also been observed in the mannan-proteins of yeast cell walls [20] and in the mannitol-containing oligosaccharides, isolated after mild alkaline borohydride treatment from rat brain proteoglycans [25]. Since the Man 1 - O - Ser/Thr linkages exist in these very distantly related organisms, we predict that they have the potential to be present in all eucaryotic organisms.

ACKNOWLEDGMENTS

The authors thank Mr Stefan Strömberg for skilful technical assistance with the carbohydrate analyses and Ms Edith Fløistrup for expert technical assistance with the isolation of Tca2-1. This investigation was supported by grants from the Swedish Medical Research Council (16X-4509).

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OLIGOSACCHARIDE STRUCTURES MEDIATING AGGLUTINATION OF SHEEP
ERYTHROCYTES BY STAPHYLOCOCCUS SAPROPHYTICUS

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ABSTRACT

Agglutination of sheep erythrocytes by Staphylococcus saprophyticus was used as a model system for adherence studies. Glycolipids were isolated from sheep erythrocyte membranes and oligosaccharides were prepared by trifluoroacetolysis. The oligosaccharides were characterized by sugar analyses, methylation analyses, gas-liquid chromatography-mass spectrometry and nuclear magnetic resonance spectroscopy. It was shown that oligosaccharides containing terminal β -D-Galp-(1-4)- β -D-GlcNAcp-(1- were good inhibitors of the hemagglutination of sheep erythrocytes by S. saprophyticus.

INTRODUCTION

Staphylococcus saprophyticus is a common cause of urinary tract infections (UTI) in young women, being second only to Escherichia coli as the most frequent causative agent (26,32,40). UTI with S. saprophyticus also occur in children (Jodal, U., personal comm. 1982) and men (10). In men such infections are most common in older age groups, particularly in those with hyperplasia of the prostate (14). That this organism may be one causative agent of nonspecific urethritis (NSU) has also been considered (15).

In approximately half of all cases with UTI caused by S. saprophyticus the upper urinary tract is involved and the concentration capacity of the kidney can be reduced (14). A proportion of all cases of UTI with S. saprophyticus seems to occur, within the same subgroup of 10-15% of all women in which the vast majority of all cases of recurrent nonobstructive UTI can be diagnosed (13).

S. saprophyticus has been shown to adhere in greater numbers to urothelial and periurethral cells than to other types of cells, e.g. skin and buccal cells (4). The organism is not known as a human pathogen in organ systems other than the urogenital tract.

The majority of strains of S. saprophyticus have, in contrast to S. aureus and to other coagulase-negative staphylococcal species, the ability to agglutinate sheep erythrocytes (12).

The capacity of bacteria and other microorganisms to

adhere to eucaryotic cells is dependent on physicochemical factors such as surface charge, hydrophobic interactions (3) and on the presence of specific carbohydrate receptors (19,20,21).

In the present investigation the agglutination of sheep erythrocytes by S. saprophyticus was used as a model system to investigate adherence properties of S. saprophyticus. Oligosaccharides were isolated by trifluoroacetylation of glycolipids from sheep erythrocyte membranes and tested as inhibitors of the agglutination. Furthermore a number of oligosaccharides and glycoproteins were subjected to hemagglutination-inhibition tests to examine the specificity of the adherence.

MATERIALS AND METHODS

Bacteria. - The eight strains of S. saprophyticus used had been isolated from voided urine specimens as described elsewhere (11). Species identification was made according to Kloos and Schleifer (17). The staphylococci were maintained on solid medium consisting of Blood agar base No. 2 (Oxoid) containing 4% defibrinated horse blood.

Erythrocytes. - One per cent suspensions of fresh, washed sheep erythrocytes were prepared in physiological saline.

Erythrocyte membranes. - Sheep blood was obtained from the slaughter house with citrate buffer as anticoagulant. Erythrocyte membranes were prepared using the method of Dodge et al. (6). The yield of lyophilized membranes was approximately

4 g per liter of blood.

Oligosaccharides. - β -D-Galp-(1-3)- β -D-GlcNAcp-(1-3)- β -D-Galp-(1-4)-D-Glc (lacto-N-tetraose) and β -D-Galp-(1-4)- β -D-GlcNAcp-(1-3)- β -D-Galp-(1-4)-D-Glc (lacto-N-neo-tetraose) were obtained from human milk as previously described (18). α -NeuNAcp-(2-3)- β -D-Galp-(1-4)-D-Glc (3'-sialyllactose) and α -NeuNAcp-(2-6)- β -D-Galp-(1-4)-D-Glc (6'-sialyllactose) were prepared from bovine colostrum by the procedure described for isolation of sialyllactoses from human milk (34).

β -D-Galp-(1-4)- β -D-GlcNAcp-(1-2)- α -D-Manp-(1-6)- β -D-Manp-(1-4)-D-GlcNAc (GM₁-pentasaccharide) and β -D-Galp-(1-4)- β -D-GlcNAcp-(1-2)- α -D-Manp-(1-3)-[- β -D-Galp-(1-4)- β -D-GlcNAcp-(1-2)- α -D-Manp-(1-6)-]- β -D-Manp-(1-4)-D-GlcNAc (GM₁-octasaccharide) were obtained from the urine of a patient with GM₁-gangliosidosis (24). β -D-Galp-(1-4)- β -D-GlcNAcp-(1-2)- α -D-Manp-(1-3)-[- β -D-Galp-(1-4)- β -D-GlcNAcp-(1-2)- α -D-Manp-(1-6)-]-D-Mannitol was prepared from human asialotransferrin by the trifluoroacetolysis procedure described for the preparation of the N-glycosidically linked carbohydrate chains in asialofetuin (28). α -D-Galp-(1-4)- β -D-Galp-(1-4)-D-Glc, β -D-GalNAcp-(1-4)- β -D-Galp-(1-4)-D-Glc and β -D-GalNAcp-(1-3)- α -D-Galp-(1-4)- β -D-Galp-(1-4)-D-Glc were produced by specific trifluoroacetolytic degradation of the glycolipids globotriaosyl-ceramide, gangliotriaosyl-ceramide and globotetraosyl-ceramide, respectively. The glycolipids were prepared from pig intestinal linings (globotriaosylceramide) (39), guinea pig erythrocyte membranes (gangliotriaosyl-ceramide) (33) and human erythrocyte membranes (globotetraosyl-ceramide) (25).

β -D-Galp-(1-4)-D-Glc (lactose) was purchased from Sigma (St. Louis, USA). β -D-Galp-(1-4)-D-GlcNAc (N-acetylactosamine) and β -D-Galp-(1-4)- β -D-GlcNAcp-(1-0)-Me (Methyl β -N-acetylactosaminide) were gifts from Dr. Göran Magnusson, Research Division, Swedish Sugar Company, Arlööv, Sweden.

The purity of all these oligosaccharides was determined by either paper chromatography or gas-liquid chromatography (as permethylated alditols). Each oligosaccharide (except the one commercial sample) were characterized by sugar analysis, methylation analysis, sequential techniques, gas-liquid chromatography-mass spectrometry and by their nuclear magnetic resonance spectra.

Glycoproteins. - Fetuin was prepared (36) from fetal calf serum and human transferrin was prepared (35) from human serum. Human antithrombin was obtained from Kabi-Vitrum, Stockholm, Sweden. The glycoproteins were desialylated by mild acid hydrolysis (37) and their purity was determined by acrylamide gel electrophoresis. The carbohydrate chains of the native and desialylated glycoproteins were analysed by sugar and methylation analysis and were in accordance with previously published structures for the carbohydrate chains in fetuin (37), human transferrin (35) and human antithrombin (8).

Analytical methods. - Sugar analyses were performed using gas-liquid chromatography (30) and mass spectrometry (2) after hydrolysis with 90% aqueous formic acid at 100°C for 5 h and also after subsequent hydrolysis with 0.25M aqueous sulphuric acid at 100°C for 18 h. Methylation analyses were performed as previously described (1).

Isolation of glycolipid fractions from sheep

erythrocyte membranes. - 230 g of lyophilized sheep erythrocyte membranes were extracted successively with 95% and then 85% aqueous ethanol (13 l of each). The combined extracts were concentrated to dryness, redissolved in 750 ml chloroform/methanol (2:1 (v/v)) and filtered. The filtrate was concentrated to dryness and dissolved in 300 ml chloroform/methanol (2:1 (v/v)) to which acetone (3 l) was added and the mixture was left at 4°C for 20 h before removing the resultant precipitate by filtration. This precipitate (32.5 g) was sonicated in 50 ml chloroform/methanol (9:1 (v/v)) and fractionated on a silicic acid column (10 x 80 cm, Silica Gel 60, Merck, Darmstadt, F.R.G.), eluted successively with 5 l chloroform/methanol (9:1, 2:1, 1:1, 1:2, 1:9, and 1:20 (v/v). A-F in Fig. 1). The fractionation was monitored by thin-layer chromatography on HPTLC plates (Merck, Darmstadt, F.R.G.) using chloroform/methanol/water (65:25:4 (v/v/v)). The glycolipids were visualized on the plates by the anisaldehyde reagent (16). The pooled fractions 1-3 (Fig. 1) were treated with 0.1M KOH in 10% aqueous methanol (100 ml/g glycolipid mixture). The vessel was flushed with nitrogen, and left with magnetic stirring in the dark at 4°C for 20 h. The alkaline hydrolysis was stopped with 2M HCl, slowly added under stirring, until pH 2-3 was reached (16). Chloroform and water were added to give a final proportion of chloroform/methanol/water of 8:4:3 (v/v/v). The phases formed were tested by thin-layer chromatography. The glycolipids in fraction 1 were mainly in the lower phase which was concentrated to dryness. Fractions 2

and 3 showed distribution of glycolipids in both phases. The material in the lower phase was collected and added to fraction 1. The material in the upper phase of fractions 2 and 3 was collected and concentrated to dryness. Fractions 1-3 were further purified by silicic acid chromatography and preparative thin-layer chromatography (Merck, Darmstadt, F.R.G.).

Nitrous acid deamination. - The dried glycolipid was dissolved in 0.5M acetic acid (1 ml acetic acid/mg glycolipid) and NaNO_2 (5 mg) was added. The solution was left at room temperature for 3 h and the excess HNO_2 was destroyed with ethylamine. The reaction mixture was degassed with nitrogen and adjusted to pH 9 with M NaOH (38). The oligosaccharides were reduced (NaBD_4) and permethylated (9).

Trifluoroacetolysis of glycolipid fractions. - The glycolipid fraction was treated with a mixture of trifluoroacetic acid and trifluoroacetic anhydride (TFA/TFAA, 1:100 (v/v), 50 ml/g glycolipid mixture) at 100°C for 48 h in a sealed tube (caution: corrosive mixture under pressure). After cooling, the reaction mixture was concentrated to dryness, dissolved in methanol (50 ml) and again concentrated to dryness. The resulting product was dissolved in glacial acetic acid (25 ml) and distilled water (25 ml) was added. After 1 h at room temperature the solution was evaporated to dryness and diethyl ether (100 ml) and distilled water (50 ml) were added. The mixture was shaken, the water phase removed and the diethyl ether phase was extracted three times with 50 ml distilled water. The combined yellowish water phase was washed three times with 25 ml diethyl ether and

evaporated to dryness and the residue was treated with 2M ammonia in 50% aqueous methanol (50 ml) at room temperature for 20 h. The solution was evaporated to dryness and the residue was acetylated using acetic anhydride and pyridine (1:1 (v/v), 50 ml, 100°C, 30 min). The acetylated product was de-O-acetylated with 2M ammonia in 50% aqueous methanol (50 ml) at room temperature (20 h). After concentration to dryness the product was fractionated on Sephadex G-15 (Pharmacia AB, Uppsala, Sweden). The fractionation was monitored by assaying the fractions for hexose (31).

Hemagglutination - inhibition tests. - Two to four hemagglutinating units (12) of bacterial suspensions in 25 µl of physiological saline were incubated at 4°C for 1 h together with 50 µl of serial twofold dilutions of the test substance in saline. To this was added 25 µl of a 1% suspension of sheep erythrocytes and the mixture was incubated at 4°C for 2 h. All tests were done in disposable, plastic microtitre plates (Linbro Ltd, England). The minimum amount of substance giving complete inhibition of hemagglutination on visual inspection was determined (12).

Abbreviations. - NeuNAc, N-acetylneuraminic acid: Gal, galactose: GalNAc, 2-acetamido-2-deoxygalactose: Glc, glucose: GlcNAc, 2-acetamido-2-deoxyglucose: Man, mannose.

RESULTS

Fractionation of the glycolipid extract gave three fractions and the major glycolipid was the Forssman antigen

(Fr 1, Fig. 1). Fraction 2 consisted of glycolipids containing 6-8 sugar residues and fraction 3 of glycolipids with more than 8 sugar residues.

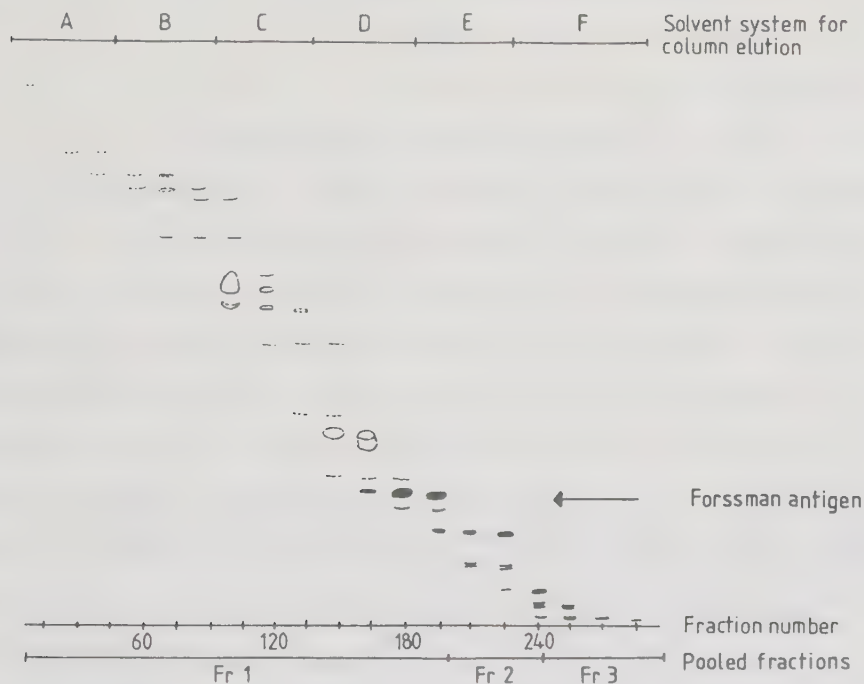


Fig. 1

Separation of glycolipids from sheep erythrocyte membranes. The plate was developed in chloroform/methanol/water (65:25:4 (v/v/v)). Spots were visualized by spraying with anisaldehyde reagent. Fractions 1-3 were pooled as indicated in the figure.

Parts of the glycolipid fractions 1-3 were trifluoroacetylated in order to specifically remove the ceramide portion of the glycolipids (29), and the liberated oligosaccharides were purified on a Sephadex G-15 column. The oligosaccharide mixtures obtained were tested for their ability to inhibit the agglutination of sheep erythrocytes by *S. saprophyticus*. The oligosaccharides from fractions 1 and 3 showed inhibitory activity. The monosaccharide composition of the oligosaccha-

ride mixtures are shown in Table 1. Fraction 1 contained

Table 1. Yields and carbohydrate composition of the glycolipid fractions prepared from 230 g of sheep erythrocyte membranes.

Fraction	Yield (mg)	<u>Relative molar proportions</u> ^a			
		<u>D</u> -Gal	<u>D</u> -Glc ^b	<u>D</u> -GlcNAc	<u>D</u> -GalNAc
1	438	1.9	1.0	0.05	1.7
2	111	0.9	1.0	0.1	0.05
3	37	1.4	1.0	1.1	0.1

^a Determined by gas-liquid chromatography-mass spectrometry after acid hydrolysis, reduction and acetylation

^b Glucose is set to 1.0

mainly D-glucose, D-galactose and 2-acetamido-2-deoxy-D-galactose and fraction 3 D-glucose, D-galactose and 2-acetamido-2-deoxy-D-glucose and trace amounts of 2-acetamido-2-deoxy-D-galactose. Methylation analysis of fraction 1 showed, inter alia, non-reducing terminal D-GalNAc, 3-O-substituted D-GalNAc, 4-O-substituted D-Gal and 4-O-substituted D-Glc which are consistent with the oligosaccharide portion of the Forssman antigen. Fraction 3 showed inter alia, the presence of non-reducing terminal D-Gal, 4-O-substituted D-Gal, 3-O-substituted D-Gal, 4-O-substituted D-GlcNAc and 3,6-di-O-substituted D-Gal by methylation analysis. Nitrous acid deamination of fraction 3 released D-Galp-(1-4)-2,5-anhydro-D-mannose. The formation of this disaccharide shows

that fraction 3 contains glycolipids with the structural element β -D-Galp-(1-4)- β -D-GlcNAcp-(1- (38).

The remaining of the glycolipid fraction 1 was further purified by silicic acid chromatography and preparative thin-layer chromatography and was divided into 3 pools (1:I-III). After release of the oligosaccharide portion by trifluoroacetolysis fraction 1:III showed inhibitory activity (Table 2). This fraction was homogen, judged by HPTLC plates, and sugar- and methylation analyses indicated it to consist of Forssman antigen. This was verified by direct probe mass spectrometry and nuclear magnetic resonance spectroscopy after release of the oligosaccharide by trifluoroacetolysis (A. Gunnarsson, J. Lundsten and S. Svensson. Acta Chem. Scand. in press). The yield of fraction 1:III was 350 mg. Fraction 3 consisted of far less material and further purification was done by preparative thin-layer chromatography. The fractionation was divided into 3 pools (3:I-III), and the oligosaccharides of fraction 3:III (12 mg) consisting of the largest glycolipids showed inhibitory activity (Table 2). The fraction was heterogeneous, but consisted of a major component (90% judged by HPTLC) and showed the same monosaccharide composition and structural elements by sugar- and methylation analyses as fraction 3.

Of the oligosaccharides tested as inhibitors of the agglutination of sheep erythrocytes by S. saprophyticus (Table 2), those containing the structural element β -D-Galp-(1-4)- β -D-GlcNAcp-(1-, inhibited the hemagglutination in concentrations of about 200-300 μ g/ml. A number of oligosaccharides lacking this structural element were inactive at

Table 2. Hemagglutination-inhibition test. Carbohydrate structures as inhibitors of the hemagglutination of sheep erythrocytes by Staphylococcus saprophyticus

Inhibitor	Minimum inhibitory concentration $\mu\text{g/ml}$
β -D-Galp-(1-4)-D-GlcNAc	300
β -D-Galp-(1-4)- β -D-GlcNAcp-(1-0)-Me	250
β -D-Galp-(1-4)- β -D-GlcNAcp-(1-3)- β -D-Galp-(1-4)-D-Glc	250
β -D-Galp-(1-4)- β -D-GlcNAcp-(1-2)- α -D-Manp-(1-6)- β -D-Manp-(1-4)-D-GlcNAc	250
β -D-Galp-(1-4)- β -D-GlcNAcp-(1-2)- α -D-Manp-(1-6)	200
β -D-Galp-(1-4)- β -D-GlcNAcp-(1-2)- α -D-Manp-(1-3)	
β -D-Galp-(1-4)- β -D-GlcNAcp-(1-2)- α -D-Manp-(1-6)	
β -D-Galp-(1-4)- β -D-GlcNAcp-(1-2)- α -D-Manp-(1-3)	200
β -D-Galp-(1-4)- β -D-GlcNAcp-(1-4)- β -D-Galp-(1-4)-D-Glc	
β -D-Galp-(1-4)- β -D-GlcNAcp-(1-4)- β -D-Galp-(1-4)-D-Glc	
α -D-Galp-(1-4)- β -D-Galp-(1-4)-D-Glc	
β -D-Galp-(1-4)-D-Gal	
β -D-Galp-(1-4)-D-Glc	
Antithrombin III (human)	
Asialoantithrombin III (human)	250
Transferrin (human)	>2500
Asialotransferrin (human)	>2500
Fetuin	250
Asialofetuin	>2500
α -NeuNAcp-(2-6)- β -D-Galp-(1-4)-D-Glc	125
α -NeuNAcp-(2-3)- β -D-Galp-(1-4)-D-Glc	>2500
Fraction 1:III	>2500
Fraction 3:III	300
	150

10 times higher concentrations. Glycoproteins containing the structural elements β -D-Galp-(1-4)- β -D-GlcNAcp-(1- in the carbohydrate chains are common in human cells and in human serum. However, in these glycoproteins the terminal β -D-Galp-(1- residue is substituted either at the 3- or 6-position by a sialic acid residue. As expected, the desialylated glycoproteins derived from antithrombin III, transferrin and fetuin were active as inhibitors whereas the native glycoproteins were inactive (Table 2). The structures of the carbohydrate chains of the glycoproteins are shown in Fig. 2.

DISCUSSION

A suspension of the majority of strains of S. saprophyticus (10^5 bacteria/ml) readily agglutinates a 1% sheep erythrocyte suspension whereas erythrocytes from humans (A, B or O), ox or guinea pig are not agglutinated by this bacterium. The agglutinin on S. saprophyticus was previously shown to be heat sensitive and susceptible to proteolytic enzymes (12) and is thus most likely of protein nature. In order to characterize the receptor structure on sheep erythrocyte membranes the total mixture of glycolipids was extracted and purified by silicic acid chromatography and thin-layer chromatography. The glycolipid fractions were then subjected to trifluoroacetolysis at conditions that released the oligosaccharides. Since these conditions also will transamidate any N-acetyl functions into N-trifluoroacetyl functions (27), O- and N-trifluoroacetyl groups were removed by

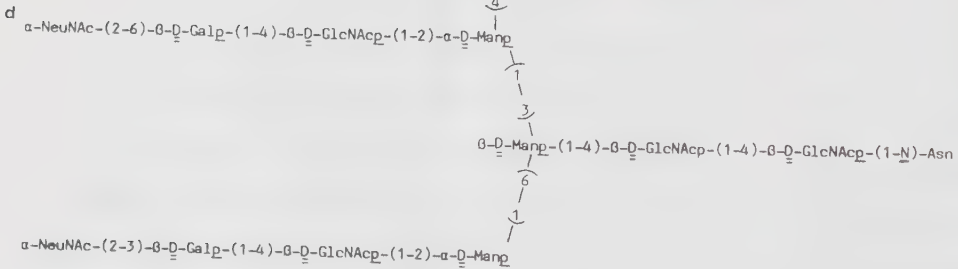
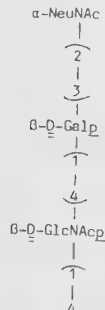
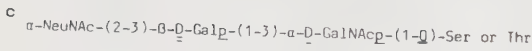
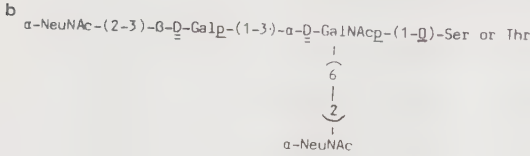
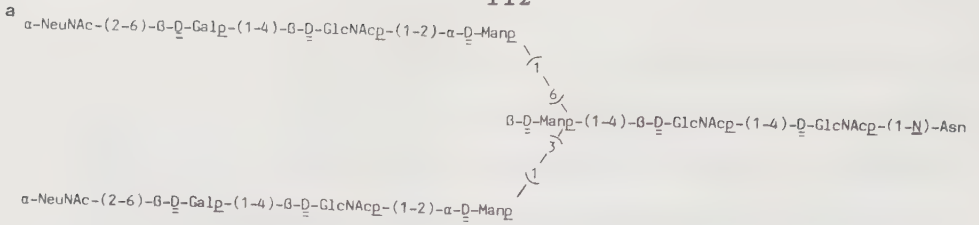


Fig. 2
Carbohydrate chains of the glycoproteins human antithrombin III and human transferrin (a) and fetuin (b, c, d).

treatment with 2M ammonia in methanol/water. Any free amino groups generated by the alkaline hydrolysis were reacylated. The oligosaccharides produced from the glycolipid fraction of the sheep erythrocyte membranes were then tested for their capacity to inhibit the agglutination of sheep erythrocytes by S. saprophyticus. It was found that an oligosaccharide mixture from glycolipid fraction 3:III was quite effective as an inhibitor (Table 2). This oligosaccharide mixture contained D-glucose, D-galactose and 2-acetamido-2-deoxy-D-glucose and methylation analysis demonstrated, inter alia, non-reducing terminal D-galactopyranose residues and 1,4-substituted 2-acetamido-2-deoxy-D-glucopyranose units.

In order to characterize the structural requirements for the adherence of S. saprophyticus we investigated a large number of oligosaccharides and glycoproteins for their ability to inhibit the agglutination of sheep erythrocytes by S. saprophyticus. As can be seen from Table 2 all oligosaccharides containing the terminal structural element β -D-Galp-(1-4)- β -D-GlcNAcp-(1- were strong inhibitors and it seems to make very little difference to what type of structure it was linked. The receptor seems to have a very specific requirement, since changing the (1-4)-linkage into a (1-3)-linkage or changing the nature of the monosaccharide units (β -D-Galp-(1-4)-D-Glc) abolishes the inhibition. The inhibitory capacity of glycoproteins (Table 2) corroborates the above findings since all glycoproteins containing oligosaccharide chains terminated with β -D-Galp-(1-4)- β -D-GlcNAcp-(1- units showed high inhibitory activity. The glycoproteins in which these terminals were substituted α -(2-3)- or α -(2-6)- with a

sialic acid residue were inactive. Conformational analysis of this type of structural unit has shown it to possess the structure depicted in Fig. 3 (22).

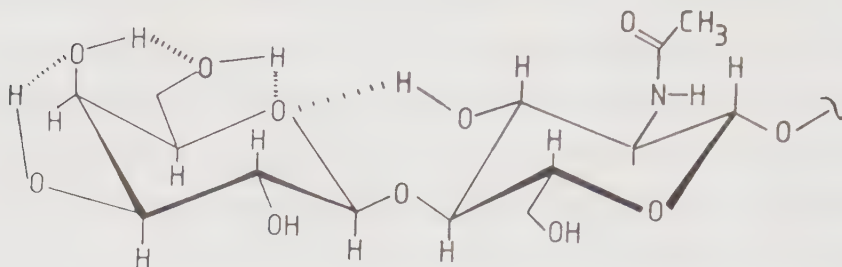


Fig. 3

Preferred conformation for β -D-Galp-(1-4)- β -D-GlcNAc-(1-

It is likely that the binding of β -D-Galp-(1-4)- β -D-GlcNAc-(1- to the combining site of is caused by a specific hydrophobic interaction. The top portion of the structural element in Fig. 3 is hydrophobic in nature if a hydrogen-bonding network involving the hydroxyl groups at C-3, C-4 and C-6 of the D-galactopyranose residue is achieved (5,22,23). Furthermore, the hydroxyl group at C-3 of the D-GlcNAc residue is expected to be hydrogen bonded to the ring oxygen of the D-Galp unit (22). Such a hypothesis of interaction based on the hydrophobicity of the disaccharide would mean that substitution at the hydrogen bonded hydroxyl groups would break the hydrogen bond network and cause the structure to lose the binding activity.

The inhibitory activity of fraction 1:III, containing the oligosaccharide portion of the Forssman antigen, α -D-GalNAc-(1-3)- β -D-GalNAc-(1-3)- α -D-Galp-(1-4)- β -D-Galp-(1-4)-D-Glc, cannot be readily explained. Conformational studies may how-

ever reveal that it possesses a hydrophobic surface similar to the top portion of β -D-Galp-(1-4)- β -D-GlcNAcp-(1-.

Agglutination of the sheep erythrocytes does not occur with other coagulase-negative, novobiocin-resistant staphylococcal species than S. saprophyticus, i.e. S. cohnii, S. sciurii or S. xylosus. The specific adhesive property is most likely a virulence factor. Oligosaccharides in the form of glycoconjugates in cell membranes of epithelial cells have been shown to act as receptors for CFA/I (colonization factor antigen I) and P-fimbriated E. coli causing urinary tract infections in man (7,19,21). The present study demonstrates the first example of bacterial adhesion to an oligosaccharide element, mediated by a bacterium lacking pili.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council (16X-4509, 3X-2-16C, 13X-4956) and from the Swedish Sugar Company.

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